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THE UNIVERSITY OF ALBERTA

Variation in *Mycena citricolor* on coffee in Costa Rica

by

Amy Wang

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

Spring 1988

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.....

Supervisor

.....

.....

Date April 15, 1988

To:

Eduardo, for being so special

My friends in the Plant Pathology Department at the University of Costa Rica

Abstract

Mycena citricolor (Berk. & Curt.) Sacc., a highly unspecialized parasite that attacks over 550 species of plants, is one of the important pathogens of coffee, causing the disease known as the American leaf spot.

Twenty-six field isolates of *M. citricolor* were studied to determine if there are differences in the behavior of this fungus among those isolated from coffee leaves in Costa Rica. Differences among isolates were found to be significant with respect to their growth rate and gemmae production when grown on agar media, oxalic acid production and mycelium dry weight when grown in broth cultures, lesion size when artificially inoculated on coffee leaves under field conditions, capacity to form the perfect stage when grown in co-culture with inducer fungi and vegetative compatibility or incompatibility. The results from vegetative compatibility or incompatibility tests provided evidence of a possible multigenic and/or multiallelic system governing heterokaryon compatibility.

Six single basidiospore cultures obtained from the basidiocarps formed by the field isolates when grown in co-culture with *Alternaria alternata* (Fr.) Keissler, and one obtained from a basidiocarp formed on a fallen leaf under field conditions, were also included in this study. All the above mentioned tests were also carried out with these cultures.

A cluster analysis was performed to integrate all the observations to group similar isolates together. The results showed that there are two well defined groups, one of them including all the single basidiospore isolates plus the isolate from *Coffea liberica* L. The rest of the field isolates from *C. arabica* and *C. canephora* formed another group. Within each group, there was variation in measured characteristics but clustering occurred at much lower levels.

All eight varieties of coffee used in this investigation were found to be susceptible to *M. citricolor* to varying degrees. Further studies on the genetics of heterokaryon compatibility in this pathogen need to be carried out, so as to facilitate breeding for resistance in coffee towards *M. citricolor*.

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Chapter I

General Introduction

A. Preface

Coffee, derived from the berries of *Coffea* L. spp., has become one of the most important beverages around the world. Its rich flavour, good colour and stimulatory effects, which are its most prominent characteristics, make it a pleasure to drink. Coffee has been, and will continue to be, one of the top five agricultural commodities in international trade (Andrade, 1987; FAO, 1984). In many countries, business with the rest of the world depends on coffee.

There are more than 125 countries that are major consumers of coffee and about one-half of these are also producers of coffee. Some of the most important coffee-producing countries are Brazil, Colombia, Costa Rica, Dominican Republic, Guatemala, Honduras, India, Indonesia, Kenya and Uganda (Wellman, 1961). In Costa Rica, 105,000 hectares, or 2% of the land is covered by coffee plantations. Coffee plays an integral role in the Costa Rican economy, representing about 34% of the total export products (Andrade, 1987).

There are many diseases that affect coffee. Of these, the American leaf spot of coffee or *ojo de gallo* incited by *Mycena citricolor* (Berk. & Curt.) Sacc., known in the past as *Omphalia flavida* Maubl. & Rangel, is one of the most important. The disease causes serious, sometimes 20 to 30%, losses. In spite of its importance, the disease has not been studied extensively. Most of the research has been oriented towards its chemical control. However, it is important to mention the contributions made by Buller (1934), Wellman (1950), Sequeira and Steeves (1954), Rodrígues (1965) and Salas and Hancock (1972) towards the understanding of the morphology, dissemination, mechanism of pathogenicity, physiology and perfect stage, respectively, of the pathogen.

Lately, the pathogen has been receiving more attention. Studies on some new aspects of biochemistry and physiology (Ayer and Dufresne, 1986; Rao and Tewari, 1987), mechanism of penetration (Tewari *et al.*, 1986) and methods of control (Curling, 1986; Mora,

1987; Solórzano, 1987; Rao and Tewari, 1988) of *M. citricolor* are being carried out at the Plant Pathology laboratories at the University of Alberta and the University of Costa Rica.

Mycena S.F. Gray appears to be more a temperate genus than a tropical one, being poorly represented under warm conditions (Dennis, 1951). Most of the species are saprophytic on mosses (*M. amicta* Fr., *M. cinerella* Karst., *M. epipterygia* Scop. ex Fr., *M. flavo-alba* Fr., and *M. metata* Fr., etc.) or decayed coniferous wood (*M. rosella* Fr., *M. alcalina* Fr., and *M. sanguinolenta* A. & S. ex Fr.) and cones (*M. strobilina* Fr., *M. strobilicola* Fav. et Kühn, *M. sperirea* Fr., and *M. seynii* Quél.) (Fries, 1949; Singer, 1975; Tomassi, 1977). Others are litter decomposers in deciduous woodlands (*M. galopus* (Pers. ex Fr.) Kummer) (Chard *et al.*, 1983) and at least two are pathogens (*M. citricolor*, *M. theobromicola* (Murr.) Dennis) (Wellman, 1950; Singer 1987). *Mycena theobromicola* has long been known to be associated with rotten cacao pods (Dennis, 1951), not as a pathogen, but as a saprophyte. However, Singer (1987) found the mushrooms of this fungus on diseased, but still attached, cacao pods, as well as on the fallen ones. The pod blackening was not thought to be due to *Monilia* Cif. & Par. or *Botryodiplodia theobromae* Pat., suggesting that *M. theobromicola* may be pathogenic on cacao. Singer (1987) conjectured that *M. theobromicola* may be the causal agent of cacao pod rot instead of *Monilia* sp. or *B. theobromae* as thought of previously.

B. American Leaf Spot of Coffee

1. General Information

The American leaf spot of coffee was discovered in Colombia by Mr. C. Michelsen in 1880 (Ernst, 1880). Soon after, Cooke (1881) identified the causal agent as *Stilbum flavidum* Cke. on coffee leaves from Venezuela and Costa Rica. After these reports, the disease was found throughout the coffee growing areas of the American Continent and it has therefore been called the "American leaf spot of coffee".

Cooke's description of the reproductive bodies caused some confusion in the classification of the fungus, and it was not until 1914 that Maublanc and Rangel described the perfect stage: a small light sulphur yellow agaric, developing on the diseased leaves of *Eriobotrya japonica* Lindl. The pathogen was then classified as a Basidiomycete, under the name of *O. flavida*. Ashby (1925), making use of some diseased coffee leaves obtained from Trinidad, proved by the pure culture method that Cooke's *S. flavidum* and the *O. flavida* of Maublanc and Rangel actually arose from the same mycelium and therefore were the same fungus. Dennis (1950), examined some samples of *Agaricus citricolor* Berk. & Curt. and found it to be the same fungus, well known to plant pathologists as *O. flavida*. He concluded that *A. citricolor* was a species of *Mycena* and suggested the name *M. citricolor* for it. This binomial is generally accepted at the present time. Taxonomically, it is a member of the Class: Basidiomycetes, Subclass: Homobasidiomycetes, Series: Hymenomycetae, Order: Agaricales, and Family: Agaricaceae.

Mycena citricolor has a very wide host range. It can be considered as an example of an unspecialized parasite. Besides coffee, there are more than 550 species of plants known to be susceptible to this pathogen (Buller, 1934; Wellman, 1972). Sequeira (1958) performed a survey of the plants commonly infected in and around coffee plantations located in the Central Plateau of Costa Rica. Plants belonging to families such as Amaranthaceae, Begoniaceae, Chenopodiaceae, Euphorbiaceae, Leguminosae, Myrtaceae and Umbelliferae from the Dicotyledons, Araceae, Bromeliaceae, Cyperaceae, Dioscoreaceae, Gramineae and Zingiberaceae from the Monocotyledons and several ferns were found to be infected.

Coffee has been considered as the only plant of significant economic importance affected by *M. citricolor*. However, there are a number of other economic plants that are susceptible to the fungus. The medicinal plants, *Cinchona officinalis* L. which is a source of quinine, and *Cephaelis ipecacuanha* L., a small shrub that contains the alkaloid emetine in its roots, are also affected. Cacao (*Theobroma cacao* L.) and even oranges (*Citrus sinensis* L.) are part of Sequeira's list, which indicates that *M. citricolor* could become a serious disease on these plants as well, provided the conditions of shade, high humidity and temperature are met

(Purseglove, 1968; Sequeira, 1958).

2. Morphology

The hyphae of *M. citricolor* are of typical basidiomycetous type, the cells being normally binucleate and having clamp connections (Sequeira, 1952).

The fungus produces fruiting bodies in two different stages: the gemmiferous stage (asexual) and the basidiocarpic stage (sexual) (Figure 1). The gemmifer, a yellow minute pin-shaped structure that serves to spread the pathogen has two parts: a slender cylindrical stalk or pedicel, which is about 2.0 mm in length, and a head or gemma of about 0.36 mm diameter, which, when fully developed, detaches readily (Buller, 1934). The gemma is shaped like an oblate spheroid (a flattened globe), with an apophysis or collar of smaller diameter. Its upper surface is slightly depressed in the centre. It consists of a central mass of relatively large pseudoparenchymatous cells surrounded above and on the sides by a thin layer of smaller and more flattened cells from which, upon germination, radiate many slender branched septate hyphae, that gives it a wooly appearance. The consistency of the whole gemmae is solid since the space between the cells is filled with a transparent mucilage (Buller, 1934).

The pedicel, which tapers gradually upwards, is a solid cylinder, quite straight and perpendicular to the substratum when young, but as it grows in length, it usually becomes more or less bent or curved. Within the apophysis, the pedicel becomes somewhat sigmoidally bent. The sigmoidal terminal portion of the pedicel is hidden from external view being inside the gemma (Buller, 1934).

The basidiocarp, which is the perfect stage of the fungus, can produce and liberate an abundance of basidiospores. The basidiocarps, which are much larger than the gemmifers, were first observed and described by Maublanc and Rangel in 1914 (translated and amended by Buller, 1934) as: "Very minute, yellowish; pileus thin, membranaceous, hemispheric-campanulate, depressed or subumbilicate in the centre, then more or less flattened, glabrous, radially striate 2.0-4.5 mm in diameter, with an acute margin, stipe

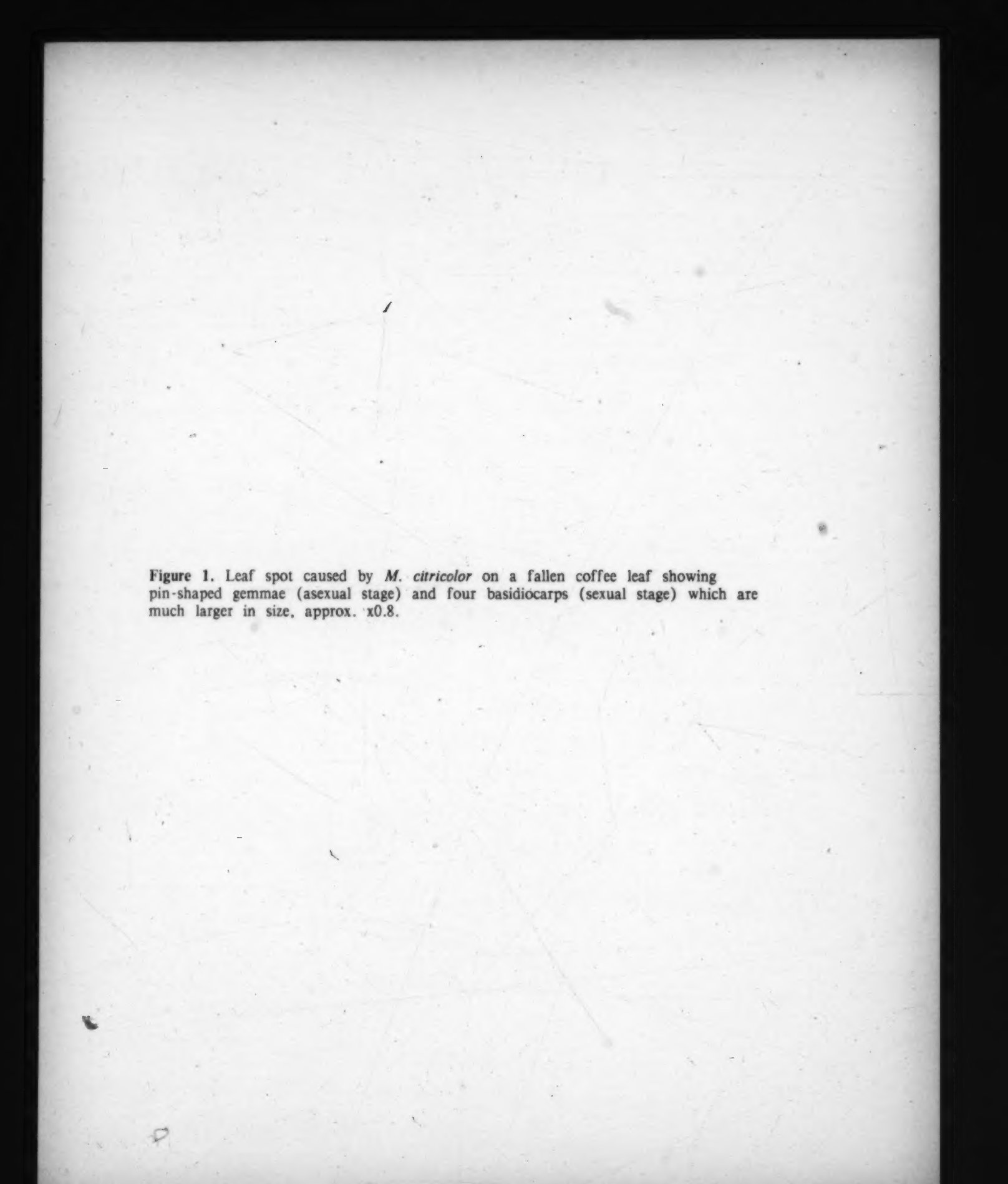

Figure 1. Leaf spot caused by *M. citricolor* on a fallen coffee leaf showing pin-shaped gemmae (asexual stage) and four basidiocarps (sexual stage) which are much larger in size, approx. x0.8.

THE QUALITY OF THIS MICROFICHE IS HEAVILY DEPENDENT UPON THE QUALITY OF THE THESIS SUBMITTED FOR MICROFILMING.

UNFORTUNATELY THE COLOURED ILLUSTRATIONS OF THIS THESIS CAN ONLY YIELD DIFFERENT TONES OF GREY.

LA QUALITE DE CETTE MICROFICHE DEPEND GRANDEMENT DE LA QUALITE DE LA THESE SOUMISE AU MICROFILMAGE.

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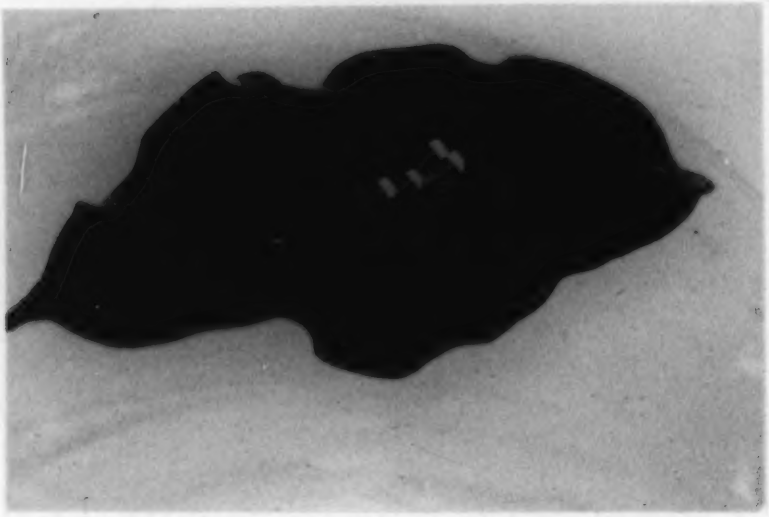

setiform, straight, thin, of the same colour, very minutely velvety. Gills few, rather distant, yellowish, somewhat waxy, triangular, attenuated at each end. Basidia clavate, 14-17.4 x 5 μ, spores minute, ellipsoid or ovoid, apiculate below, hyaline, eguttulate or with one guttule, 4-5 x 2.5-3 μ.".

The basidiocarps (Fig. 1) are not found frequently under field conditions. Carvajal (1939) found them on fallen diseased leaves in association with the gemmae but Wellman (1950) searched for them, without any success, for over a period of two years under many different conditions in Costa Rica, Guatemala and, Colombia. In 1972, Salas and Hancock performed a detailed study on the effect of some saprophytic fungi on the induction of basidiocarp formation in *M. citricolor*. They found that several species of *Penicillium* Link ex Fr. were able to act as inducers, but *P. oxalicum* was especially superior. Some other fungi, such as *Cladosporium* Link ex Fr. sp., *Alternaria* Nees ex Wallr. sp., and *Phycomyces blakesleeanus* Kunzē were also inducers but to a lesser extent. From this work, they concluded that under field conditions basidiocarp formation by *M. citricolor* is dependent upon the activity of saprophytic fungi. Wellman (1972) considered that light and repeated washing of the diseased tissue by rain or dew are also necessary.

Buller and Vanterpool (1926) discovered the pale bluish-white bioluminescence of the mycelium of *M. citricolor* when they were examining a bread culture (one part of bread: four parts of water) in the dark room. On examining the leaf spots, the luminosity was not confined only to the mycelium in the leaf, but extended also to the gemmae. This light emission was present only when the mycelium was actively growing and ceased when it had become old.

Airth *et al.* (1966) showed that fungal bioluminescence reactions include: (a) either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), (b) an electron acceptor, (c) soluble dehydrogenase(s), (d) molecular oxygen, and (e) the particulate enzyme luciferase. A two step reaction was thought to take place: (1) the reduction of dehydro- or oxyluciferin ('X') by NADH or NADPH in the presence of the soluble dehydrogenase(s), and (2) the light emitting oxidation

of 'X'H$_2$ by molecular oxygen with the particulate enzyme luciferase. In the case of *M. citricolor*, Kubawara and Wassink (1966) found that the unknown luciferin or dehydroluciferin can be extracted only with hot water, that it is not soluble in acetone, chloroform, diethyl ether, diethyl ether-ethanol (3:1), *n*-butanol or benzene, and that it can produce light with the fungal enzyme system (enzymatic bioluminescence) or non-enzymatically upon addition of sodium hydroxide and hydrogen peroxide. However, they were not sure if what they found can be considered as the fungal luciferin or not, since more information, like molecular weight, quantitative organic tests and determination of functional groups were required.

According to Wellman (1972), as early as 1878 the luminescence of young, still active spots of *M. citricolor* on coffee was observed during the night and this gave the spots the Spanish name of "candelillas" (little candles).

3. Symptoms and Dissemination of the Pathogen

The disease is characterized by the formation of small spots (usually 6-10 mm in diameter) on the leaves. These lesions are generally circular in outline but sometimes become ovate due to limitation by veins, or somewhat irregular when two or more lesions fuse. The young lesions are dark (Fig. 2a) and the older ones are lighter in colour (Fig. 2b). During the past few years, E. Vargas (unpublished information) has noticed the presence of some old lesions that, instead of showing the characteristic creamy-white colour, were grayish-black or reddish. Sometimes the lesions provide sites of entry to other tissue destroying microorganisms producing spots of larger sizes. With the advent of dry season, the diseased tissue may fall away, leaving circular openings in the leaf (Fig. 2b, Buller, 1934; Wellman, 1950). The round shape of the lesions has given the disease, the Spanish name *ojo de gallo* or rooster's eye.

If infection takes place on the middle vein, close to the leaf base, it causes petiole epinasty in the younger leaves, and premature drop in the case of older leaves. The leaves are able to sustain infections in quite large numbers before falling, if no large veins are infected.

Figure 2. Leaf spots caused by *M. citricolor*. **(a)**. Young lesions starting to show gemmae (arrowhead) **(b)**. Old lesions. Note the presence of a circular opening (arrowhead) left by breaking of the diseased tissue during the dry season.

a

b

In other words, defoliation is not so much dependent on spot numbers as on their location on the leaf (Wellman, 1950). The effect of disease on yield is due mainly to defoliation. Because the trees are weakened, the leaves are not soon replaced, but the disease never kills the tree (Sequeira and Steeves, 1954).

The fungus can attack young stems where it causes scars and so weakens the points affected which are easily broken off by wind. On berries (Figure 3), it produces a slight discoloration on the seed but there is no evidence of seed transmission of the pathogen (Buller, 1934; Wellman, 1950).

Under very moist climatic conditions, on the upper surface of the spots and also, to some extent on the lower surface, gemmae develop. Once mature, they are detached by the action of rain drops and are carried to nearby leaves. The gemmae usually settle on their upper surfaces and readily become attached to the leaf by means of a mucilagenous substance. Numerous infection hyphae penetrate through the epidermis and attack the internal leaf tissues producing a spot. That the gemmae are the most important reproductive structures is demonstrated by the facts that they are produced in considerable numbers, are easily detached from their pedicels, are small so that they are readily dispersed, contain mucilagenous substance that attaches them to the leaf and produce infection hyphae from all around the outer side of the oblate spheroidal part of each gemma (Buller, 1934).

4. Epidemiology

Studies carried by Prof. E. Vargas and his group at the University of Costa Rica (unpublished information) and by Bonilla (1979a) in El Salvador, have shown that the disease development follows a seasonal fluctuation closely related to rainfall and relative humidity.

Shortly after the rainy season starts, the number of diseased leaves and lesions per leaf begin to increase. It takes around one week for gemmae to be formed. Rain or heavy dew prepares them for detachment from the pedicels and rain drops carry them to nearby plants. Wellman (1972) found that gemmae can germinate on the moist surface of a leaf within one hour and that wind has little effect on gemmae dispersal because of their large size

Figure 3. Coffee berries heavily infected by *M. citricolor* showing gemmae.

and weight.

In Costa Rica, maximum infection occurs in September - October which coincides with the peak of the rainy season. Spotting levels begin to decline in December, reaching the lowest levels in February, March, April and early May. December through May are the driest months, but heavy dew may occur in certain areas, allowing some infection to occur. The infection levels rise steady from May - August.

The requirement of shade by coffee plants is a popular belief considering that natural coffee grows in the forest under shade. Most of the plantations in Mexico and Central America were established under these conditions, using plants of banana and *Inga* L. spp. as sources of shade (Figure 4). The trees used for shade intercept light and restrict wind movement. Consequently, the humidity inside the plantation increases. Under these circumstances, infection by *M. citricolor* is more severe and heavy defoliation due to the disease may take place (Andrade, 1987; Wellman, 1972).

In the last decade, Costa Rica started to increase the area of coffee grown in the sun, without shade, with excellent results (Figure 5). This has placed the country among the highest in productivity around the world (Andrade, 1987). As expected, infection by *M. citricolor* was drastically reduced in these plantations. However, recent surveys have shown that the pathogen is becoming adapted to the new conditions. The lesions, instead of being creamy-white, are reddish and can be found on leaves totally exposed to the sun (E. Vargas, unpublished information).

5. Physiology

a. Effect of light on gemmae formation

The stimulatory effect and/or requirement of light in fungal reproduction has been shown for several fungi such as *P. blakesleeanus* (Bergman, 1972; Bergman *et al.*, 1969), *Trichoderma viride* Pers. ex Fries. (Gressel and Hartman, 1968) and *Nectria haematococca* Berk. & Curt. (Curtis, 1972). The action spectra for photoinduced sporulation in these

Figure 4. Well shaded coffee plantation. Under these conditions, infection by *M. citricolor* can be very severe. A coffee seedling nursery is seen in the foreground.

Figure 5. Coffee plants being grown in full sunlight. Under these conditions, *M. citricolor* infection is greatly reduced, although recent observations seem to indicate that the fungus is becoming adapted to this condition.

4

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fungi are very much alike, with an action band between 400 and 500 nm (blue light), suggesting that the photoreceptor pigment involved may be similar in all cases. The nature of the photoreceptor molecule is not clear, but the resemblance of these action spectra to the absoption spectra of rivoflavin and some carotenoids indicates that these are the possible molecules (Presti and Delbruck, 1978). Upon absorption of light by the photoreceptor(s), metabolic pathways must subsequently be altered towards fruiting body formation (Moore-Landeker, 1982).

Buller (1934) observed that cultures of *M. citricolor* kept in the dark grew very well but did not produce any gemmae in the course of several weeks. If these cultures happened to be exposed to daylight, numerous gemmae appeared, not only on the mycelium used as inoculum, but also on the new growth. Furthermore, exposure to light for an hour or less was enough to stimulate gemma formation. From the above observations, he concluded that the formation of gemmae is initiated by the stimulus of light.

Rodrígues (1965) studied the effect of light on gemmae formation and reported that the maximum number of gemmae and large amounts of yellow pigment were formed when cultures of *M. citricolor* were grown under near ultraviolet light (310-400 nm). Preliminary characterization of the yellow pigment produced in the gemmae indicated that it might be a flavonoid. However, since the pigment is formed in the gemmae and both seem to be formed at the same time, there is no evidence that this pigment is the photoreceptor necessary to trigger the chain of reactions involved in gemmae formation. Lesions on leaves were also affected by light. Inoculated leaves kept in light developed gemmae 9 or 10 days after inoculation while lesions on leaves kept in the dark continued to enlarge and did not form gemmae (Rodrígues and Arny, 1966).

b. Metabolic studies

Sequeira and Steeves (1954) reported for the first time that defoliation caused by *M. citricolor* was not a result of mechanical injury to the leaf but was due to the inactivation of a plant growth hormone of the host by the pathogen. In fact, one lesion located on the middle

vein close to the petiole causes the leaf to drop, whereas mechanical injury at the same place did not cause any appreciable acceleration of abscission. Application of indolacetic acid (IAA) to the petiole below the lesion delayed leaf drop considerably, which suggested that the fungus prevented the normal flow of auxin from the leaf blade into the petiole. This auxin inactivating substance was present in the culture filtrate, as well as in the extracts of ground mycelium. They suggested that an oxidative enzyme was responsible for the main pathogenic effect of *M. citricolor*.

Ray (1956) and Ray and Thimann(1956) confirmed the presence of an oxidative enzyme. They determined its properties but were not able to identify it. Ray continued to work with the enzyme and in 1960, he suggested that it could be a peroxidase. Rodrígues (1965) also detected the presence of an indolacetic acid oxidase using infected coffee disks. Since this enzyme is extracellular, he suggested that it may diffuse out of the diseased area, and be responsible for the premature abscission of infected coffee leaves.

The presence of perfect tetrahedral calcium oxalate crystals scattered in the culture medium below the colonies of *M. citricolor*, was first reported by Ashby in 1925. Sixty years later, Rao *et al.* (1985) rediscovered the same crystals which were present not only in the culture medium, but also at the site of infection and in the necrotic lesion tissue. These crystals were identified as being those of calcium oxalate. After determining the capacity of the fungus to produce oxalic acid, a key role in the pathogenesis by *M. citricolor* was suggested for this acid (Rao and Tewari, 1987). Oxalic acid sequesters calcium from the host as calcium oxalate, resulting in disintegration of the infected tissue. Because application of solutions of sulfuric acid of different concentrations also produced similar lesions, it seems that lesion development may, at least partially, be the result of lowered pH brought about by oxalic acid secretion by the pathogen. Also, by lowering the cell pH, some enzymes may become more active, such as indolacetic acid oxidase, cellulase and polygalacturonase that can contribute to further tissue disintegration.

Additional work has shown that oxalic acid production was evident as early as four days after incubation of the fungus in a coffee cell wall medium, but no activity of the

enzymes polygalacturonase and cellulase were detectable at this time, and only small levels of enzyme activities were evident eight days after incubation. Therefore, these results indicate that oxalic acid plays a major role in the pathogenesis of *M. citricolor* by sequestering the calcium from host cell wall pectates while macerating enzymes play a relatively minor role in tissue disintegration. The lesion morphology of *M. citricolor* correlates well with these findings as the diseased tissue exhibits no or little evidence of maceration (D.V. Rao and J.P. Tewari, unpublished information).

Citricolic acid is another metabolite reported from the culture filtrates of *M. citricolor* (Ayer and Dufresne, 1986). This compound is thought to be a degraded steroid and its functional significance is not known.

6. Control

One of the first chemicals found to have some effect in controlling the disease was the Bordeaux mixture, however it was economically and physically impossible to use under the coffee plantation conditions at that time (Carvajal, 1939).

Full defoliation of severely diseased trees was suggested by Wellman (1951) as an alternative method of disease control but heavily pruned plants required two or more years to start producing once again.

Copper containing fungicides continued to be used even though they did not seem to be effective in certain areas during the peak of the rainy season. Soon, more chemicals were tested. Mercury compounds were found to inhibit the production of gemmae in the leaf spots under laboratory conditions (Echandi, 1956). Similar results were obtained in the field. Unfortunately, toxic mercuric residue was detected in the harvested coffee beans (Echandi and Segall, 1958).

Lead arsenate was suggested, as early as 1952, as a possible method of controlling this pathogen. In 1957, Castaño officially recommended its use in Colombia based on the excellent results obtained in field trials and the apparently non-translocation of the toxic compounds to the beans. Other countries started to test the fungicide under their conditions with similar

results (Bianchini *et al.*, 1958; Salas, 1960; Díaz and Napoles, 1961). Soon it became widely used, while testing for the presence of arsenate in the beans continued. Pereira and Echandi (1964) found both lead and arsenic in coffee leaves and beans, but levels in beans, even at the higher concentrations of spray, were below the tolerance limit established by the United States Food and Drug Administration at that time.

Alternative methods of control have been tested over the years due to the high toxicity of the eradicant fungicide lead arsenate (Bonilla, 1978; Bonilla 1979b; Leandro and Soto, 1980). In some countries like Costa Rica, there are strict regulations concerning its commercial use. By legislation, spraying activities must be conducted during the months of January and June. For the purchase of this product, an authorization must be issued by a registered agronomist to the proven owner of the plantation (Morales and Jaramillo, 1982).

Biological control of *M. citricolor* using *Trichoderma* spp. has proved to be successful under experimental conditions (Arroyo, 1975; Curling, 1986, Paez, 1976; Vargas, 1984).

Since oxalic acid produced by *M. citricolor* sequesters calcium from the host cell walls, external application of this element to neutralize the toxic effects of the acid proved to be a good method of disease control under laboratory conditions. The results obtained by Rao and Tewari (1988) suggest that calcium hydroxide may potentially be used to control the disease under field conditions. Field experiments being carried by E. Vargas and his group (unpublished information) in Costa Rica are currently providing some encouraging information in this direction.

C. Natural variation in fungi

Genetic variation is a necessary condition for evolution to take place. In fact, when examining any natural population, it becomes evident that the morphological and physiological characteristics of the individuals within a species are not fixed, but may vary from one individual to another. In case of fungi, these differences become evident when they are isolated or subcultured using single spores or hyphal tips (Burnett, 1976).

One of the most important sources of variation is the sexual process. Through segregation and recombination of genes during the meiotic process, variation is introduced in the progeny. However, this is not the only source of variation, since all plants and pathogens produce variants by means of mutation and perhaps, by means of cytoplasmic inheritance, and in the case of fungi, heterokaryosis and parasexualism also takes place (Agrios, 1978).

1. Cytoplasmic inheritance

Cytoplasmic inheritance, seems to occur in all plants and pathogens except those microorganisms where cytoplasm is absent. It is the acquisition , through extrachromosomal inheritance, of certain characteristics, mainly physiological processes, which were not present before (Agrios, 1978). These hereditary determinants, occurring outside the chromosomes can be transmitted sexually or asexually and are capable of self-reproduction. In certain cases, their phenotypic expression may also depend upon the genotype (Esser and Kuenen, 1967).

In the case of pathogens, some of the adaptations due to cytoplasmic inheritance are: tolerance to previously toxic substances, utilization of new substances for growth, and changes in their virulence towards host plants. On the other hand, the plants themselves can also inherit, through cytoplasm, resistance to infection by certain pathogens (Agrios, 1976).

It has been known for a long time that when conjugation, a sexual-like process in bacteria takes place, DNA is transfered from the donor to the recipient cell by cell-to-cell contact. The genes to be transferred can be parts of non-chromosomal DNA arranged in a circular molecule called a plasmid or can be a block of genes in the chromosomes (Lewin, 1985; Snyder *et al.*, 1985). The presence of plasmid-like molecules has also been reported in fungi. Two strains of *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. that infect the crucifers, *Brassica oleracea* L. and *Raphanus sativus* L., show mitochondrial plasmid-like DNA's that may carry information necessary for pathogen specialization (Kristler *et al.*, 1987; Kristler and Leong, 1986). Because plasmids are capable of replicating independently within the host cells, and are inherited in a regular way as those cells multiply, they can be used to incorporate certain characteristics into a pathogen, such as hypovirulence (Havir and

Anagnostakis, 1983, 1985). Since plasmids are not dependent upon chromosomes, there can be exchange of plasmids between different species or even genera, thus incorporating different characteristics in the species (Novick, 1980). Since many microbes have plasmids which can be exchanged between strains of the same species or different species, it is entirely possible that they may be responsible for at least some variation, observed among the various microbial isolates.

2. Heterokaryosis

Heterokaryosis is a condition in which cells of fungal hyphae contain nuclei that are genetically different. This condition may be brought about by mutation. Since the nuclear population in any mycelium is usually very large, the chances of mutation, in at least one genetic locus of some nuclei, are very high. However, these new nuclear types are usually low in frequency, so that they may be undetectable on a gross level. Heterokaryosis can also develop as a result of hyphal anastomosis. In this case, heterokaryons are present in the mycelium derived from the zone of fusion. Finally, heterokaryosis may be a result of spore formation, where there is inclusion of different nuclei in a single spore (Burnett, 1976; Davis, 1966; Raper, 1968).

Under natural conditions, heterokaryon formation is responsible for a great deal of variation in fungi. Phenotypically and physiologically, the heterokaryotic mycelium can be quite different from its parental types, since its morphology and physiology is determined by the interaction of all the genetically different nuclei and the cytoplasm. The interaction of these nuclei turns out to be more important when they can complement each other by compensating for the deficiencies of the other. Under these circumstances, the chances of survival increase due to the availability of a large genetic pool from which to draw material for adaptation (Moore-Landeker, 1982).

Heterokaryosis can substitute, to a certain extent, the sexual phase. However, variability, in the absence of sexual recombination, is limited by the nuclear types that are available and the mutations derived from them (Davis, 1966).

3. Parasexual cycle

Parasexualism is the mechanism by which recombination can take place based not on sexual reproduction, but on mitosis. It is a sequence involving heterokaryon formation, fusion of genetically dissimilar nuclei and recombination and segregation of the diploid chromosomes at mitosis (Agrios, 1978; Davis, 1966; Moore-Landeker, 1982).

The parasexual cycle resembles sexual reproduction. In both cases, unlike hyphae (or modifications thereof) may fuse (plasmogamy) and their nuclei will occupy the same cell. These unlike nuclei fuse (karyogamy) to form a diploid (zygote) nucleus. However, with the parasexual cycle recombination takes place during mitosis followed by haploidization (but without meiosis). The recombinant haploid nuclei appear among vegetative cells, which differ genetically from those of the parent mycelium (Moore-Landeker, 1982; Roper, 1966).

Both heterokaryosis and the parasexual cycle are very important for those fungi that have no sexual reproduction. These cycles provide for somatic variation in the vegetative phase of their life cycles. This is also true for those fungi where the sexual phase is present, although in this case, additional and significant variation is incorporated through the sexual reproduction (Roper, 1966).

D. Objectives of the Thesis

The objectives of this thesis were to determine if there is variation in the isolates of *M. citricolor* among those isolated from diseased coffee leaves in Costa Rica, and to group similar isolates together, based on their growth rate and gemmae production when grown in artificial media, capacity to form the perfect stage, vegetative compatibility or incompatibility, oxalic acid production, and lesion development. The reaction of some coffee cultivars to artificial inoculation with the pathogen was also assessed, as well as, the capacity of the basidiospores to infect coffee leaves.

Chapter II

Materials and Methods

A. Isolates

Mycena citricolor was isolated on potato dextrose agar (PDA) from diseased coffee leaves collected from the principal coffee growing areas in Costa Rica (Table 1). A hyphal tip from each isolate was transfered to a clean PDA plate and these cultures were used throughout this work.

The PDA used was prepared as follows: 200 g of potatoes were washed and sliced without peeling, placed in a container with 500 mL of distilled water and boiled until they became soft. The liquid was filtered through a cheesecloth, adjusted to the initial volume, and 20 g of dextrose was added to it. In a boiling water bath, 18 g of agar was melted in 500 mL distilled water. The melted agar was mixed with the potato-dextrose broth and autoclaved for 15-20 minutes at 120 C (1.1 kg/cm^2 pressure).

For further growth and maintenance of the isolates, the PDA was supplemented with 0.2% of yeast extract. Stock cultures were stored at 4 C.

All 33 isolates listed in Table 1 were used in all the experiments unless otherwise indicated. The first 26 isolates (MCH2 to MCH17) were obtained from diseased leaves, the remaining seven were monosporic cultures obtained from basidiocarps developed by some of the field isolates under laboratory conditions. The last isolate (ssF) was a single basidiospore culture from a field-collected mushroom on a fallen leaf of coffee.

B. Colony size and gemmae production

The isolates were grown on two types of PDA: home-made (described above) and Difco PDA. In both cases, 0.2% yeast extract was added.

Each isolate was grown in five plastic petri plates (100 X 15 mm) containing approximately 25 mL of each medium. A 5 mm plug from the margin of an actively growing culture was placed in the centre of each plate. The plates were incubated at 25 C under

Table 1. Isolates of *M. citricolor* used in this study.

	Isolate ident. number[a]	*Coffea* sp. host	Place of origin	Colour of lesion
1	MCH2[b]	*C. arabica*	Heredia	Tan
2	MCH3[b]	*C. arabica*	Turrialba	Tan
3	MCH4[b]	*C. arabica*	Sabanilla	Black
4	MCH5a[b]	*C. arabica*	Puriscal	Tan
5	MCH5b[b]	*C. arabica*	Puriscal	Tan
6	MCH5c[b]	*C. arabica*	Puriscal	Tan
7	MCH6a[b]	*C. arabica*	Sabanilla	Tan
8	MCH6b[b]	*C. arabica*	Sabanilla	Tan
9	MCH6c[b]	*C. arabica*	Sabanilla	Tan
10	MCH6d[b]	*C. arabica*	Sabanilla	Tan
11	MCH7[b]	*C. arabica*	Heredia	Black
12	MCH8a[b]	*C. arabica*	Los Santos	Tan
13	MCH8b[b]	*C. arabica*	Los Santos	Tan
14	MCH8c[b]	*C. arabica*	Los Santos	Tan
15	MCH8d[b]	*C. arabica*	Los Santos	Tan
16	MCH9a[b]	*C. canephora*	Turrialba	Tan
17	MCH9b[b]	*C. canephora*	Turrialba	Tan
18	MCH9c[b]	*C. canephora*	Turrialba	Tan
19	MCH10[bc]	*C. arabica*	Heredia	Tan
20	MCH11[bc]	*C. arabica*	San Carlos	Tan
21	MCH12[bc]	*C. arabica*	San Carlos	Red
22	MCH13[bc]	*C. arabica*	Turrialba	Tan
23	MCH14[bc]	*C. arabica*	Turrialba	Tan
24	MCH15[bc]	*C. arabica*	Turrialba	Red
25	MCH16[bc]	*C. arabica*	Turrialba	Tan
26	MCH17[bc]	*C. liberica*	Turrialba	Tan
27	ssMCH5a[d]			
28	ssMCH9b[d]			
29	ssMCH10[d]			
30	ssMCH11[d]			
31	ssMCH13[d]			
32	ssMCH14[d]			
33	ssF[e]		Turrialba	

[a] The letters M, C, and H stand for *Mycena*, *citricolor* and *hoja* (Spanish for leaf).
Same number followed by different letter indicates that the isolates were collected from the same plantation.
ss = single spore.

[b] From infected coffee leaves.

[c] Kindly supplied by Prof. Edgar Vargas, Plant Pathology Laboratory, University of Costa Rica.

[d] Single basidiospore isolates obtained from basidiocarps developed under laboratory conditions.

[e] Single basidiospore isolate obtained from a field collected basidiocarp.

continuous lighting of approximately 31 $\mu Em^{-2} sec^{-1}$.

After seven days, two orthogonal measurements of colony diameter were made. The average of the two was regarded as the colony diameter. Gemmae production in the same cultures was determined by cutting off two pieces of 1/8 of each colony (from opposite sides) and placing the individual pieces in 50 mL polyallomer tubes containing 5 mL tap water. These tubes were stirred for 15 seconds with a Vortex-genie mixer to release the gemmae. The gemmae were collected on Whatman #1 filter paper and counted with the help of a stereomicroscope. The total number of gemmae per plate was then calculated.

Analysis of variance (ANOVA) was performed on the average diameter measurements of the colonies and on the total numbers of gemmae formed on the plates on each medium. Treatment means were compared using Tukey's test. The correlation between these two variables was determined.

C. Oxalic acid production and mycelium dry weight

Preliminary tests were carried out to determine the best culture medium and incubation period. Two kinds of media were used: Difco potato-dextrose broth with 0.2% (w/v) yeast extract (PDB) and a synthetic liquid medium recommended by Sequeira (1954) which contained: glucose (20.0 g), ammonium tartrate (9.2 g), potassium dihydrogen phosphate (5.8 g), magnesium sulfate (2.5 g), ferric chloride (1% solution, 0.6 mL), thiamine hydrochloride (100.0 μg) and distilled water (1000.0 mL).

One hundred and twenty-five mL Erlenmeyer flasks containing 50 mL of each medium were inoculated with ten 3 mm hyphal plugs of *M. citricolor* and placed on a shaker at 120 rpm under normal diurnal light conditions in the laboratory (approximately 5.5 $\mu Em^{-2} sec^{-1}$ and 22 C). After 10 days, oxalic acid production and mycelium dry weight were recorded for each of the five replicates per medium.

To determine the optimum incubation period, a time course experiment was carried out using four isolates chosen at random and grown in PDB. Samples were taken every two days over a 14-day period. Changes in pH values were also recorded.

For the 33 isolates studied, mycelium dry weight was determined using 12-day-old cultures grown on PDB. They were individually filtered through a tared Whatman #1 filter paper moistened with distilled water, and the paper with the mycelium was dried at 85 C for 24 hours. Dry weight was recorded for each of the five replicates per isolate.

Oxalic acid isolation and determination was done using the culture fluid from the above experiment. It was carried out according to the method of Bateman and Beer (1965). Ten mL of filtrate were incubated overnight with 5 mL of 0.1 M calcium chloride-acetate buffer, pH 4.5 and centrifuged at 16,300 g for 10 minutes in a Sorvall refrigerated centrifuge (RC-5B). The pellet was resuspended in 10 mL of 5% acetic acid saturated with calcium oxalate, centrifuged as above and finally the sediment was washed with 10 mL distilled water. After centrifugation, the pellet was dissolved in 50 mL of 4N H_2SO_4 and titrated at 90 C against 0.1N $KMnO_4$. Calculations were made as grams of oxalic acid per liter of broth.

An analysis of variance was carried out with the mycelium dry weight and oxalic acid production data. Isolate means were compared using Tukey's test. The correlation between the two variables was also determined.

D. Induction of the perfect stage

Penicillium sp., *Talaromyces flavus* (Klöcker) Stolk & Samson and *Alternaria alternata* (Fr.) Keissler, all laboratory contaminants, were found to be inducers of basidiocarp formation.

A preliminary test, using three randomly chosen isolates of *M. citricolor* was performed to determine the best inducer. *Mycena citricolor* was grown on one side of a PDA petri plate and the fungus to be tested as the inducer, on the opposite side. Inocula of both fungi consisted of 5 mm plugs from the actively growing margin of the colonies on PDA. Control treatment consisted of *M. citricolor* grown alone. Cultures were exposed to normal diurnal light conditions in the laboratory. For each fungus tested as an inducer, three replicates were used. The data, collected one month after both fungi were inoculated, consisted of the number of basidiocarps formed per replicate.

The best inducer was grown in co-culture with all the isolates of *M. citricolor* as described above, using five replicates, and the whole experiment was performed three times.

Single basidiospore isolates were obtained by attaching the basidiocarps to the lower side of the lid of water agar plates. The basidiospore germination was initiated in 24 to 36 hours. Single germinated basidiospores, observed under the stereomicroscope, were transferred to PDA plates.

Young, fully expanded coffee leaves were used for inoculation with the basidiospores. The detached leaves were placed on top of a 5 mm wire mesh in a petri plate (150 x 20 mm) to avoid direct contact with distilled water that was placed underneath. Inoculation was performed on the upper and lower surfaces of five leaves, with and without injury. In case of wounded leaves, epidermis of the interveinal areas of both halves was scratched with a hypodermic needle. In all cases, the basidiocarps were attached to the lower side of the lid and placed over one-half of the leaf. The opposite half served as control. Leaves were incubated at 22 C and 5.5 μEm^{-2} sec^{-1} for two weeks.

E. Vegetative compatibility or incompatibility

Field and monosporic isolates were paired in every possible combination by placing 5 mm hyphal plugs 2-3 cm apart on PDA plates. Cultures were exposed to normal diurnal laboratory conditions and 22 C.

The morphology of the zone of interaction was studied four weeks after both the isolates were inoculated. This experiment was performed three times.

F. Lesion size and reaction of some coffee varieties to artificial inoculation

Field work was carried out during July, August, September and October, 1987 when the rainy season was well established. The forenoons were sunny but rain started to fall soon after mid-day and continued during the afternoon and part of the night.

The plants used for inoculation were part of CICAFE's (Coffee Research Centre) germplasm collection. The farm is located at Barva, province of Heredia, at 1,054 m above

mean sea level, with an average annual precipitation of 2,115 mm. (For detailed meteorological data, see Appendix 1).

Four commercial varieties: (Villalobos, Bourbon, Caturra and Catuaí Rojo) and four promissory selections of the hybrid Catimor (#8657, 8660, 8664 and 8667) with resistance towards coffee rust (*Hemileia vastatrix* Berk. & Br.) were used for inoculation with all the isolates of *M. citricolor*, except for ssMCH10 and ssF which never produced gemmae.

Three healthy and vigorous plants of each variety were selected and five leaves belonging to the second or third pair of leaves from the tip on different branches from the middle third portion of each plant were used.

In order to get enough gemmae of each isolate to inoculate the leaves, two-week-old cultures grown of PDA were cut into small pieces and transferred to several 40 mL, clear glass, wide mouth bottles with screw caps, containing 10 mL of PDB supplemented with 1 mL of sterilized *A. alternata* culture fluid. Gemmae formation was highly stimulated and the cultures were ready to be used in two weeks.

The leaves selected for inoculation were first sprayed with distilled water. The interveinal area of the upper leaf surface was scratched with a hypodermic needle at three points on the right half and two on the left. Four of these points were inoculated with 3-5 gemmae (Figure 6a). The remaining one served as control. After placing the gemmae, individual leaves were covered with a white plastic bag (10 X 12 cm) (Figure 7b) containing a piece of moistened paper towel. The bag protected the gemmae from being washed away by the rain and the moistened paper towel provided enough humidity for the infection hyphae to start their growth. The plastic bags were removed after one week and the leaves were allowed to remain attached to the plant for another 7 days. After detachment, two orthogonal diameters of each lesion were measured and the mean was taken as the lesion diameter.

Due to manpower restrictions, only half of the varieties were inoculated with one isolate in a single day. The remaining plants were inoculated the day after.

Since the isolate ssF never produced gemmae, a preliminary test to determine the capacity of this isolate to infect coffee leaves using mycelial discs was performed under

Figure 6. Artificial inoculation of coffee leaves. The selected leaves were first sprayed with distilled water and 3-5 gemmae (arrowhead) were placed over a preinjured spot (a). After inoculation, individual leaves were covered with a white plastic bag (b).

a
b

laboratory conditions. Young, fully expanded coffee leaves from the variety Caturra were used for inoculation. The detached leaves were placed on top of a 5 mm wire mesh, in a clear plastic box with cover, to avoid direct contact with the wet plastic foam that was placed underneath. Inoculation was performed on the upper surface of injured leaves by placing 5 mm mycelial discs over one-half of the leaf. The opposite half served as control. Leaves were incubated at 5.5 $\mu Em^{-2} sec^{-1}$ and 22 C for two weeks.

Analysis of variance was performed on the average lesion diameter measurements of the 15 leaves per treatment (31 isolates over the eight varieties).

G. Cluster analysis

Cluster analysis was used to determine if isolates could be grouped, based on the measured characteristics. It was not performed on the discrete variable compatibility or incompatibility. Results from the mushroom induction test were not included because one-half of the isolates did not produce them and also from the practical point of view that they are seldom formed under field conditions. The field inoculation experiment on different varieties took about four months to complete due to constraints on manpower. During this period, there may have been minor variations in the environmental conditions. Therefore, the lesion size data on different varieties was also excluded from the cluster analysis.

Chapter III

Results

A. Colony size and gemmae formation

Growth of isolates, measured as colony diameter, was greater at P=0.05 on PDA prepared with fresh potatoes than on Difco PDA, but the general morphology of the colony for any single isolate was the same on both media. All the isolates were characterized by radial growth of fine hyphae from the plug used as inoculum. The great majority of isolates formed white mycelial mats close to the agar surface with scanty aerial hyphal growth. In more than 50% of the isolates, the colonies reached over 6 cm diameter in 7 days of incubation at 25°C (Figure 7a-d). A small number of isolates grew rather slowly and formed a smooth, almost translucent or watery white sparse mycelial mat over the surface of the medium (Figure 7e and f). The diameters of these colonies were usually around 2-5 cm after 7 days of growth at 25°C. Intermediate types between these two phenotypes were also present.

During the first 2 or 3 days of growth, pale yellow gemmae initials started to appear as small dots on the plug, and then on the older parts of the growing colony. Within about two days, the gemmae initials completed their development, giving rise to bright yellow gemmae.

On each individual medium, differences among isolates appeared to be significant (Table 2).

Unlike growth, the total number of gemmae produced by each isolate were very similar on both media. However among isolates, the variations were very large, ranging from zero (e.g. MCH8c and ssMCH9b) to 6912.8 (MCH5a) gemmae on Difco PDA and 0 to 5324.0 on home-made PDA, respectively (Table 3). Most of the single basidiospore cultures did not produce any gemmae regardless of the size of the colony.

The correlation coefficient between colony size and gemmae production was low (0.4) although significant at P=0.05.

Figure 7. Colonies of *M. citricolor* on PDA after 5 days of incubation at 25°C under continuous lighting of approximately 31 μEm^{-2} sec^{-1}. **a**: MCH3, **b**: MCH9c, **c**: MCH10, **d**: MCH8c, **e**: MCH5a, **f**: MCH5c, approx. x0.5.

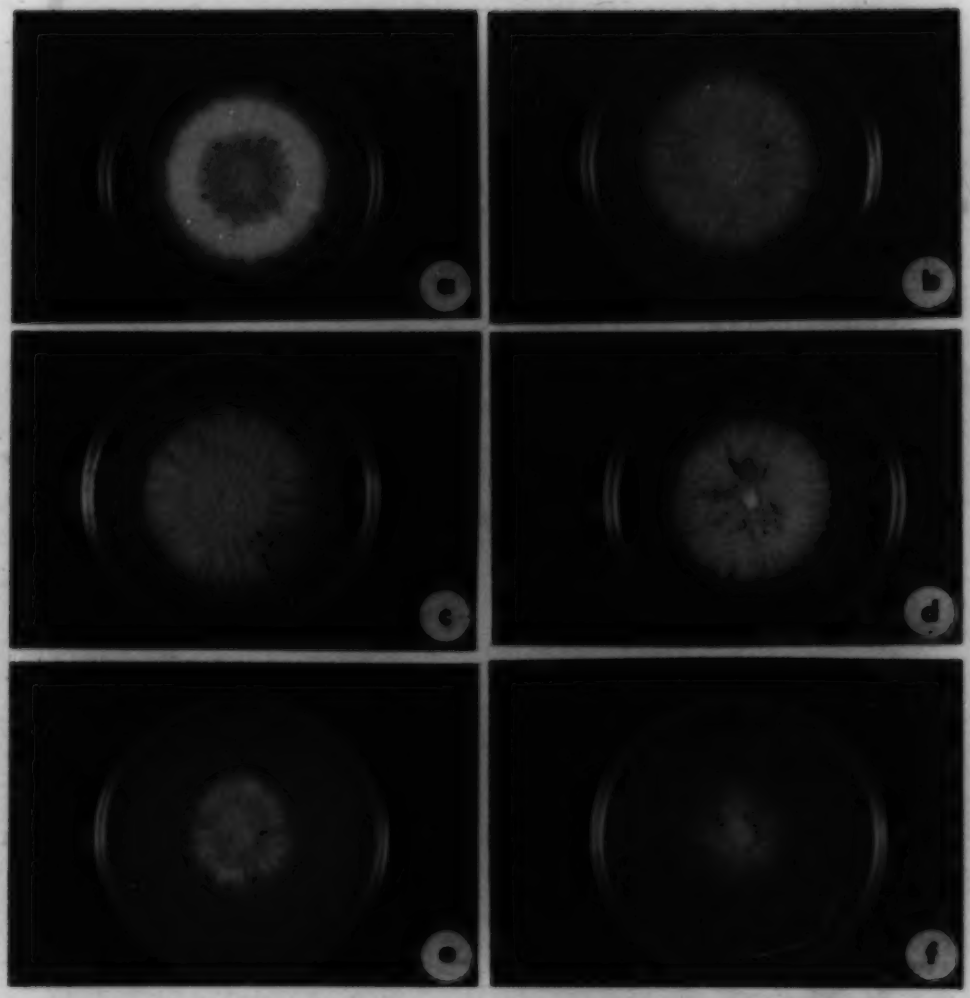

a
b
c
d
e
f

Table 2. Colony diameter (cm) of *M. citricolor* after seven days of growth on two agar media at 25°C and 31 $\mu Em^2 sec^{-1}$.

Isolate	Difco PDA	Isolate	Home-made PDA
MCH6c	7.65 a*	MCH10	7.32 a*
MCH6d	7.52 ab	MCH12	7.28 a
MCH10	6.96 ab	MCH7	7.18 a
MCH7	6.86 ab	MCH2	7.16 a
MCH4	6.82 ab	MCH6b	6.88 ab
MCH8b	6.76 ab	MCH6a	6.74 ab
MCH12	6.74 ab	MCH4	6.70 ab
MCH2	6.72 ab	MCH6c	6.68 ab
MCH3	6.63 ab	MCH6d	6.67 ab
MCH9c	6.58 ab	MCH11	6.62 abc
MCH8c	6.57 ab	MCH3	6.59 abc
MCH6b	6.40 ab	MCH9b	6.58 abc
MCH6a	6.38 ab	MCH9c	6.58 abc
MCH8a	6.33 ab	MCH16	6.58 abc
MCH16	6.26 ab	MCH14	6.44 abc
MCH5a	6.21 ab	MCH15	6.44 abc
MCH8d	6.21 ab	MCH8a	6.26 abc
MCH9b	6.18 ab	MCH8c	6.21 abc
MCH9a	6.07 abc	MCH5c	6.20 abc
MCH14	5.85 abcd	MCH8d	6.20 abc
MCH5c	5.76 abcd	MCH9a	6.08 abc
MCH15	5.70 abcd	MCH13	5.94 abc
MCH5b	5.61 abcd	MCH8b	5.93 abc
MCH11	5.61 abcd	MCH5b	5.84 abc
MCH13	5.14 abcde	MCH5a	5.70 abcd
ssF	4.13 abcde	ssF	5.37 abcde
MCH17	3.93 abcde	MCH17	4.86 abcdef
ssMCH13	3.87 bcde	ssMCH13	4.13 abcdef
ssMCH9b	2.42 cde	ssMCH5a	3.73 bcdef
ssMCH5a	2.39 cde	ssMCH11	3.31 cdef
ssMCH10	2.31 de	ssMCH10	2.45 def
ssMCH11	2.24 de	ssMCH9b	2.29 ef
ssMCH14	1.79 e	ssMCH14	1.95 f

* Each value represents a mean of five replicates.
Values followed by the same letter are not significantly different at P =0.05.

Table 3. Gemmae production by *M. citricolor* after seven days of growth on two agar media at 25°C and 31 $\mu Em^2 sec^{-1}$.

Isolate	Difco PDA	Isolate	Home-made PDA
MCH5a	6812.8 a*	MCH5a	5324.0 a*
MCH14	4048.0 b	MCH9a	4236.0 ab
MCH9a	4008.8 bc	MCH5c	3853.6 abc
MCH5c	3875.2 bc	MCH14	3853.6 abc
MCH6b	3648.2 bc	MCH9b	3713.6 bcd
MCH9c	3316.0 bcd	MCH15	3701.6 bcd
MCH6a	3282.4 bcde	MCH9c	3348.0 bcd
MCH9b	3208.6 bcdef	MCH13	3336.8 bcd
MCH13	3130.0 bcdefg	MCH6a	3129.6 bcd
MCH5b	2658.4 bcdefg	MCH5b	3076.4 bcd
MCH16	2198.4 cdefgh	MCH6b	3038.4 bcd
MCH6c	2038.4 defghi	MCH8d	2917.6 bcd
MCH15	1874.4 defghi	MCH16	2633.2 cd
MCH3	1820.4 defghij	MCH6c	1038.4 de
MCH6d	1696.4 efghij	MCH6d	1037.6 de
MCH7	1860.8 fghijk	MCH11	808.8 e
MCH8d	1189.6 ghijk	MCH3	643.2 e
MCH12	1163.2 ghijk	MCH7	624.0 e
MCH10	840.8 hijk	MCH2	547.2 e
MCH2	761.6 hijk	MCH17	443.2 e
MCH11	688.8 hijk	MCH12	414.4 e
MCH17	287.6 ijk	MCH10	348.0 e
ssMCH5a	216.8 jk	MCH4	226.0 e
MCH14	213.6 jk	ssMCH11	196.0 e
ssMCH14	146.0 jk	ssMCH14	101.6 e
MCH8a	126.4 jk	ssMCH5a	85.6 e
MCH8b	62.4 k	MCH8a	22.4 e
MCH8c	0.0 k	MCH8b	0.0 e
ssMCH9b	0.0 k	MCH8c	0.0 e
ssMCH10	0.0 k	ssMCH10	0.0 e
ssMCH11	0.0 k	ssMCH9b	0.0 e
ssMCH13	0.0 k	ssMCH13	0.0 e
ssMCHF	0.0 k	ssMCHF	0.0 e

* Each value represents a mean of five replicates.
Values followed by the same letter are not significantly different at P = 0.05.

B. Oxalic acid production and mycelium dry weight

Preliminary tests to determine the best culture medium between PDB and the synthetic broth for oxalic acid production, showed that PDB was superior to the chemically defined one. The amount of oxalic acid (g/L) accumulated in PDB after 10 days of growth was about three times and the mycelium dry weight twice of those in the synthetic medium (Table 4).

The time course experiment using four randomly chosen isolates of *M. citricolor* showed that oxalic acid concentration in the culture broth mostly followed the growth curve of the fungus. It reached peak levels after 12 days of incubation after which it decreased, even though the mycelial growth continued slightly or leveled-off (Figures 8 and 9). The pH of the medium showed a different behaviour, and dipped to 4.0 - 4.1 after 2 to 6 days of incubation from its initial value of 5.3 - 5.4. The pH drop was slowest in MCH2 and took about 6 days to reach the aforesaid range (Figure 10).

The various isolates exhibited a great deal of variation with respect to oxalic acid production. After 12 days of growth on PDB, the oxalic acid concentration ranged from 0 (ssF) to 2.8 g/L (MCH13) with most of the isolates showing between 1 and 2 g/L. Besides MCH13, only three other isolates (MCH7, MCH9b and MCH12) produced more than 2 g/L. On the other hand, all the single basidiospore isolates produced less than 1 g/L of oxalic acid (Table 5).

Table 4. Oxalic acid production and mycelium dry weight of *M. citricolor* after 10 days of growth on two liquid media.

Medium	Oxalic acid (g/L)	Mycelium dry weight (g/L)
Synthetic broth	0.43 a*	6.73 a
PDB	1.35 b	13.38 b

*Each value represents a mean of five replicates.
Values followed by the same letter are not significantly different at P=0.05.

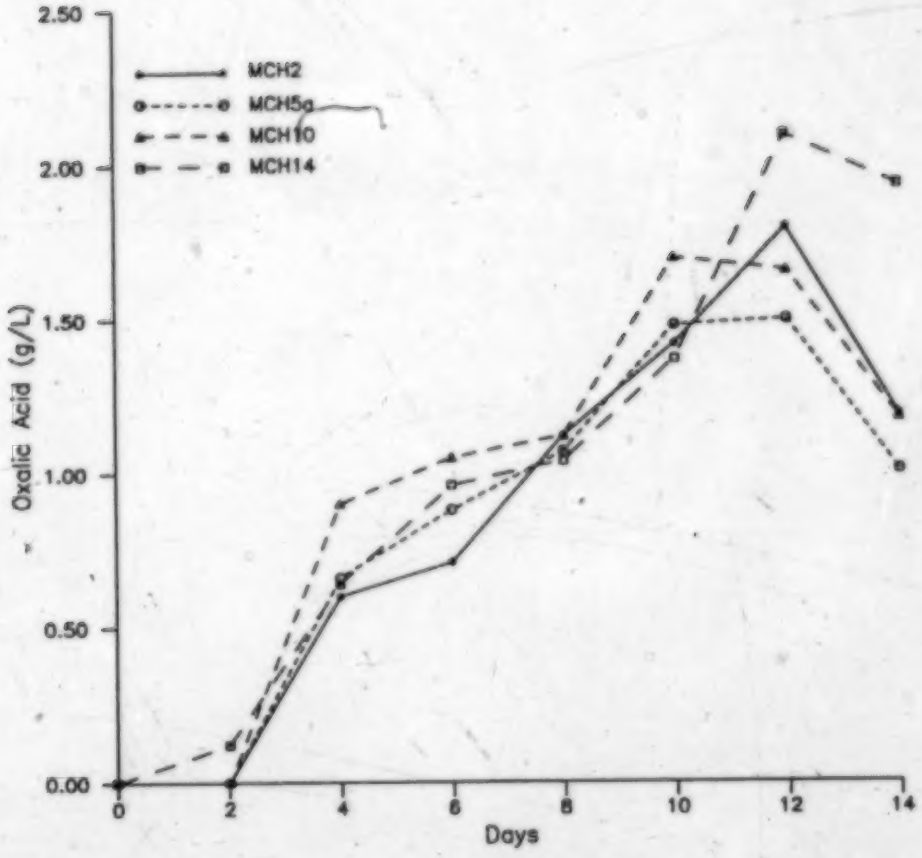


Figure 8. Time course of oxalic acid production by four isolates of *M. citricolor* in PDB.

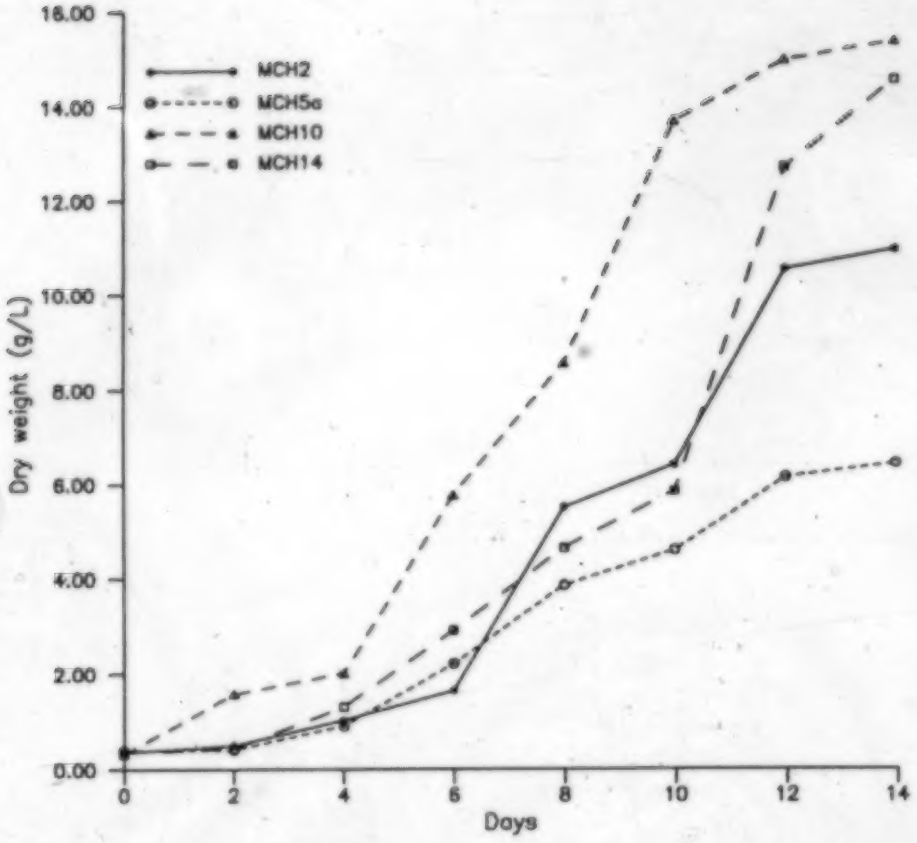


Figure 9. Time course of mycelial growth in four isolates of *M. citricolor* in PDB.

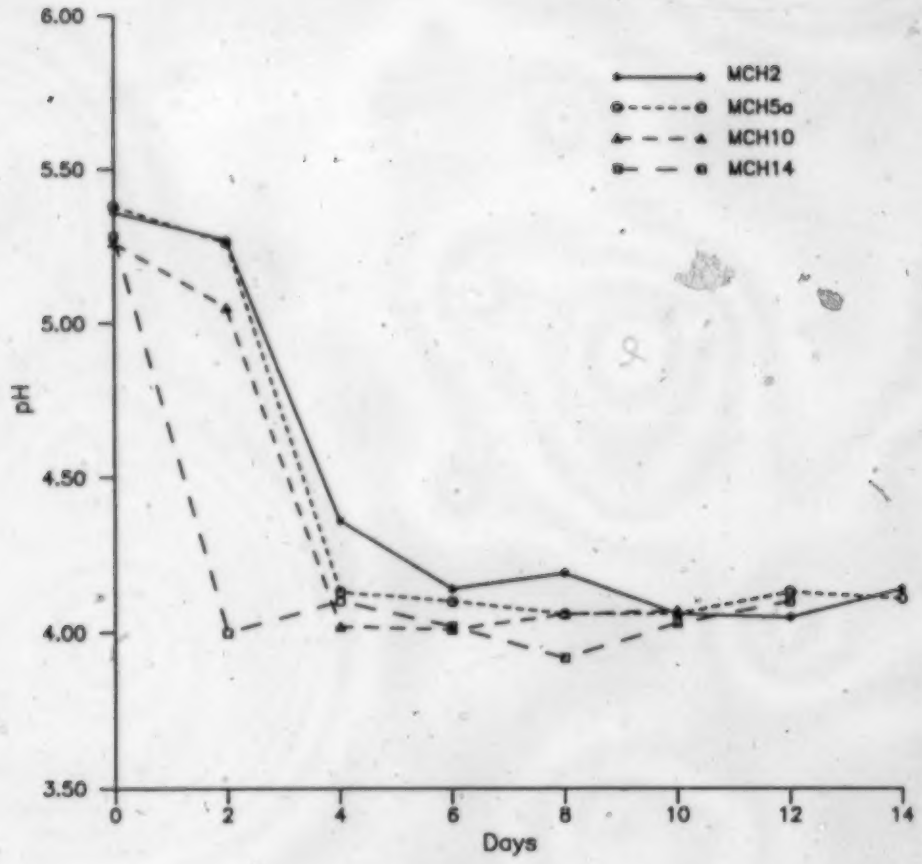


Figure 10. Time course of pH levels in PDB inoculated with *M. citricolor*.

Table 5. Oxalic acid production and mycelium dry weight of *M. citricolor* after 12 days of growth on PDB at 22 °C and 5.5 μEm^2 sec^{-1}.

Isolate	Oxalic acid (g/L)	Isolate	Mycelium dry weight (g/L)
MCH13	2.8500 a*	MCH2	15.9960 a*
MCH12	2.5300 ab	MCH6a	15.4120 ab
MCH7	2.4400 abc	MCH8d	15.7980 ab
MCH9a	2.0300 bcd	MCH9c	15.3080 abc
MCH3	1.8780 cde	MCH7	13.7480 abcdd
MCH14	1.8000 cdef	MCH6c	13.7140 abcde
MCH8a	1.7780 def	MCH9b	13.0980 abcde
MCH15	1.7140 def	MCH15	12.5060 abcdef
MCH9c	1.5820 defg	MCH6d	12.1740 abcdefg
MCH16	1.5440 defgh	MCH9a	12.1380 abcdefg
MCH4	1.4460 defgh	MCH11	11.7040 abcdefg
MCH8b	1.4240 defghi	MCH13	11.4040 bcdefg
MCH11	1.4100 defghi	MCH12	11.2780 bcdefgh
MCH5c	1.2980 defghi	MCH5b	11.0700 cdefghi
MCH6d	1.3800 efghi	MCH17	10.8180 defghij
MCH8c	1.3640 efghij	MCH5c	10.8180 defghijk
MCH5a	1.3560 efghij	ssMCH10	10.7220 defghijk
MCH6c	1.3020 efghij	MCH10	10.0640 defghijk
MCH5b	1.2980 efghijk	MCH8b	9.9700 defghijk
MCH6b	1.2660 efghijk	MCH6b	9.9180 defghijk
MCH2	1.1240 fghij	MCH4	8.3180 efghijk
MCH6a	1.0420 ghijkl	MCH8a	8.8800 efghijkl
MCH9b	0.9680 ghijklm	MCH3	8.3180 fghijklm
MCH10	0.9000 hijklm	MCH8c	8.2000 fghijklm
MCH8d	0.7580 ijklmn	ssMCH5a	7.1540 ghijklm
ssMCH13	0.7100 jklmn	MCH14	6.9500 hijklmn
ssMCH10	0.6000 klmn	MCH5a	6.7680 ijklmn
ssMCH9b	0.5520 klmn	MCH16	6.6040 ijklmn
ssMCH14	0.5400 klmn	ssMCH13	5.1120 klmn
MCH17	0.4820 lmn	ssMCH11	4.5740 lmn
ssMCH11	0.4500 lmn	ssF	3.7300 mn
ssMCH5a	0.3980 mn	ssMCH9b	3.1160 m
ssF	0.0000 n	ssMCH14	1.6080 n

* Each value represents a mean of five replicates.
Values followed by the same letter are not significantly different at P = 0.05.

The correlation coefficient between oxalic acid and mycelium dry weight was 0.33, which is not significant at the 5% level. Isolates that exhibited a good growth did not necessarily produce high amounts of oxalic acid. The mycelium dry weight for MCH2 was almost 16 g/L after 12 days of growth and the amount of oxalic acid produced was only around 1.12 g/L. MCH13 produced more than double oxalic acid (2.85 g) but its mycelium dry weight was 11.4 g after the same period of time.

C. Induction of the perfect stage

Penicillium sp., *T. flavus* and *A. alternata* were found to be inducers of the sexual stage of *M. citricolor*. Preliminary tests showed that when grown in co-culture, the basidiocarps were produced near the zone where the two fungi made contact (Figure 11). No basidiocarps were formed when *M. citricolor* was grown in monoculture.

The capacity of inducer fungi to stimulate the formation of basidiocarps in *M. citricolor* is variable. Among the three fungi tested, *A. alternata* was the best inducer, followed by *Penicillium* sp. and *T. flavus*, being in the third place. The average number of basidiocarps formed by *M. citricolor* isolates when grown in co-culture with *A. alternata* was significantly higher compared to the number of basidiocarps formed when *Penicillium* sp. or *T. flavus* were used as inducers (Table 6).

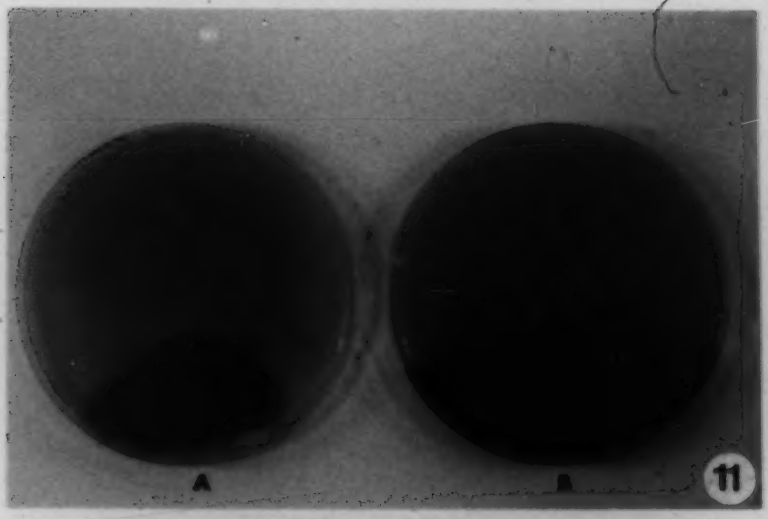
Table 6. Average number of basidiocarps formed in *M. citricolor* when grown on PDA after 8 weeks in co-culture with inducer fungi.

	Average number of basidiocarps		
Inducer	MCH5b	MCH10	MCH15
A. alternata	56.0 a*	69.3 a	20.2 a
Penicillium sp.	8.2 b	12.6 b	16.3 b
T. flavus	0.0 c	3.2 c	0.4 c

*Each value represents a mean of three replicates.
Values followed by the same letter are not significantly different at P=0.05.

Figure 11. Co-cultures of *Mycena citricolor* (isolate MCH9b) with *Penicillium* sp.**(a)** and *A. alternata* **(b)** showing basidiocarp formation on PDA after 3 weeks of growth, approx. x0.5.

Figure 12. Basidiocarp formation in *M. citricolor* (isolate MCH10) when grown in co-culture with *A. alternata* on PDA after 3 weeks of growth, approx. x0.55.


A
B
11

12

Furthermore, the basidiocarp-induction response in *M. citricolor* due to *A. alternata* was evident shortly after both fungi were grown together (2 weeks); with *Penicillium* sp., the basidiocarps started to form after 20 days and finally, the response in *T. flavus* was noticeable only six weeks later.

The response of the 26 isolates of *M. citricolor* to *A. alternata* can be summarized as follows:

a. Isolates that formed fertile mushrooms: MCH5a, MCH9b, MCH10, MCH11, MCH12, MCH13 and MCH14.

b. Isolates that formed sterile mushrooms: MCH2, MCH3, MCH4, MCH5b, MCH6b, MCH6c, MCH9c and MCH15.

c. Isolates that did not form mushrooms: MCH5a, MCH6a, MCH6d, MCH7, MCH8a, MCH8b, MCH8c, MCH8d, MCH9a, MCH16 and MCH17.

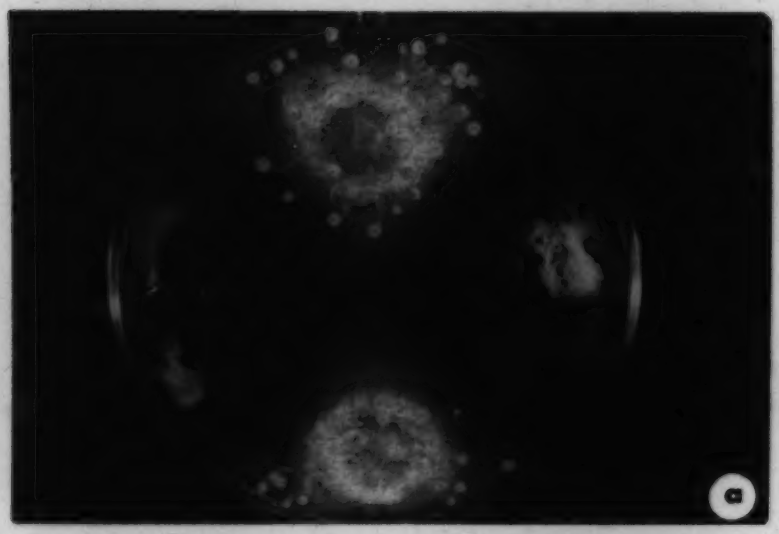
None of the seven single basidiospore cultures formed basidiocarps when grown in co-culture with *A. alternata*.

Alternaria alternata not only induced mushroom formation (Figure 12), but also estimulated, to a certain extent, gemma production, specially near the zone where the two colonies met. *Alternaria alternata* also showed some activity as a growth inhibitor of *M. citricolor*. With a few isolates of *M. citricolor*, a clear zone was formed between the two fungi (Figure 13a). With others, this inhibition zone was not prominent, but gemmae and mushrooms that were formed close to *A. alternata* colony gradually became necrotic (Figure 13b).

When *A. alternata* was grown on PDB, the basidiocarp inducing factor was also present in the culture fluid. Attempts to identify this substance are being carried out by Prof. W.A. Ayer of the Department of Chemistry at the University of Alberta. So far, it is known that the inducing factor is resistant to autoclaving and can not be extracted into organic solvents.

Basidiospore production was abundant in the fertile mushrooms. Single basidispore cultures from different and from the same isolate of *M. citricolor* exhibited a great deal of

Figure 13. Inhibitory effects of *A. alternata* on growth of *M. citricolor* on PDA after 3 weeks. In some cases, an inhibition zone was formed between the two colonies like in MCH10 **(a)**, in some others (e.g. MCH5c), gemmae and mushroom that were growing close to *A. alternata* gradually turned necrotic (arrowhead)**(b)**.


a

b

variation (Figure 14). One randomly chosen culture from each isolate of *M. citricolor* was included in this study. Microscopic examination of the single basidiospore cultures revealed that the hyphae of two of them (ssMCH13 and ssMCH14) were similar to those of the field isolates in the sense that they showed clamp connections. The hyphae of the remaining five single spore cultures (ssMCH5a, ssMCH9b, ssMCH10, ssMCH11 and ssF) had no clamp connections.

When wounded and unwounded coffee leaves were inoculated on the upper and lower surfaces with basidiospores from each of the isolates that formed basidiocarps, no infection took place.

D. Vegetative compatibility or incompatibility

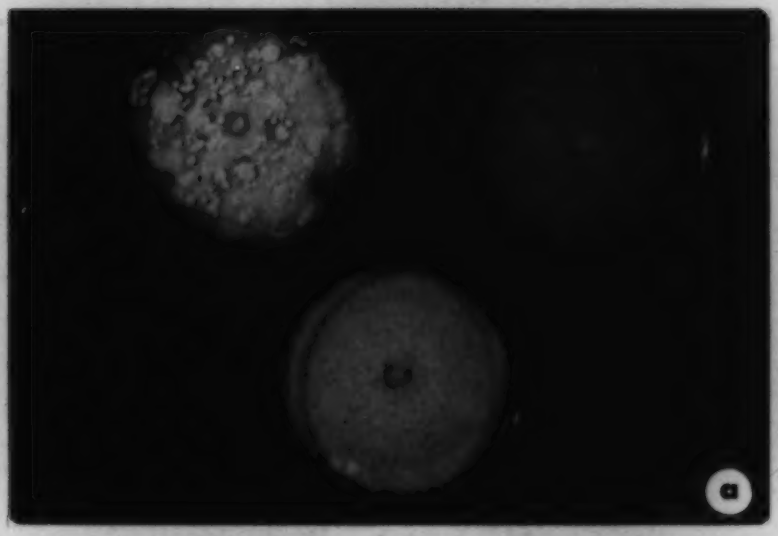
Pairing of isolates in all possible combinations resulted in three different types of reactions: compatible, incompatible and an intermediate type.

Compatibility was observed almost exclusively when isolates were paired with themselves. There was no interaction zone and the mycelia intermingled freely (Figure 15) An incompatible reaction was recognized by the formation of a clear area in the region of contact of the mycelia, with scattered hyphal growth between the two colonies (Figure 16). In the third type of reaction, the two colonies grew very close to each other without showing any inhibition zone. In this intermediate type of reaction, each isolate appeared to maintain its individual integrity (Figure 17).

From a total of 528 pairings (not including self-pairings), only 17 (3.2%) were compatible, 159 (30.1%) incompatible and the remaining 352 (66.7%) showed intermediate reaction (Table 7).

Attempts to group the isolates into interaction groups, where within a group the paired isolates grew together and no antagonism zones developed, was unsuccessful. The reaction of the isolates was not consistent. For instance, MCH14 was compatible with MCH3 and MCH15, but when MCH3 and MCH15 were paired, an intermediate reaction was observed. This type of behavior was observed with most of the isolates (Table 7), so it was

Figure 14. Single basidiospore isolates from the basidiocarps formed by **(a)** MCH9b and **(b)** MCH10 grown on PDA for 7 days. Note a great deal of variation among cultures, approx. x0.38.


a

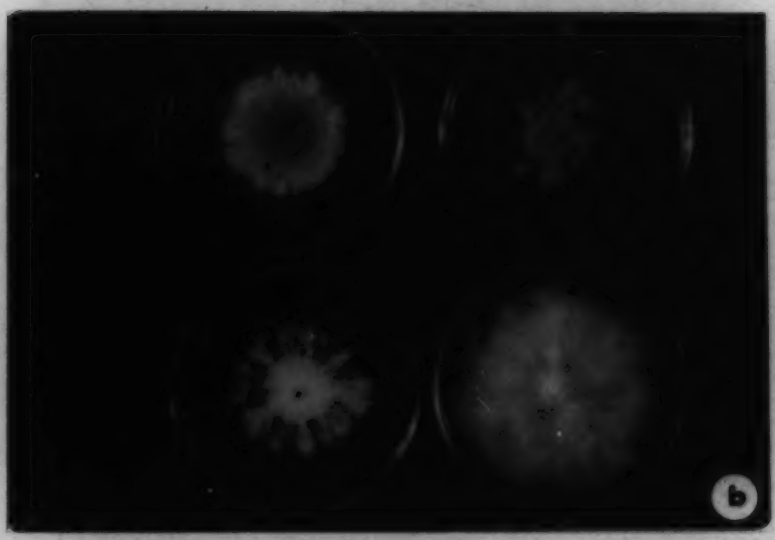
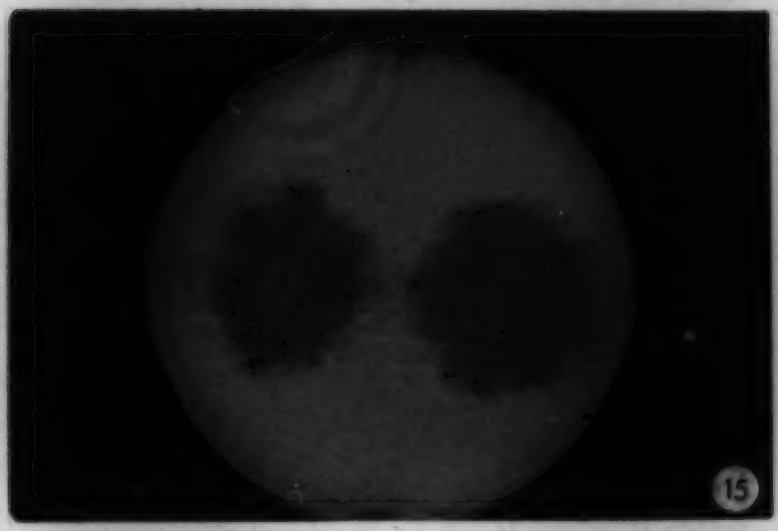

b

Figure 15-17. Hyphal interaction and antagonism among field isolates of *M. citricolor* after 4 weeks of growth on PDA. **15.** Compatible reaction in the self-pairing of MCH2; the mycelia intermingled. **16.** Incompatible reaction between two isolates (MCH3 and MCH6a); an inhibition zone was formed between the two colonies, approx. x0.75. (continued on next page)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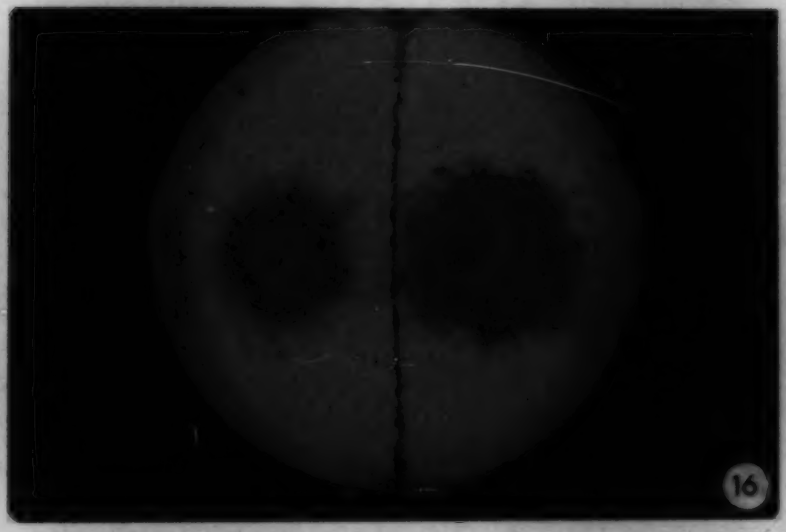
16

Figure 15-17. Hyphal interaction and antagonism among field isolates of *M. citricolor* (continued). 17. Intermediate reaction between two isolates (MCH8b and MCH10). The colonies met at the centre of the plate and grew very close together, but no inhibition zone was observed, approx. x0.75.

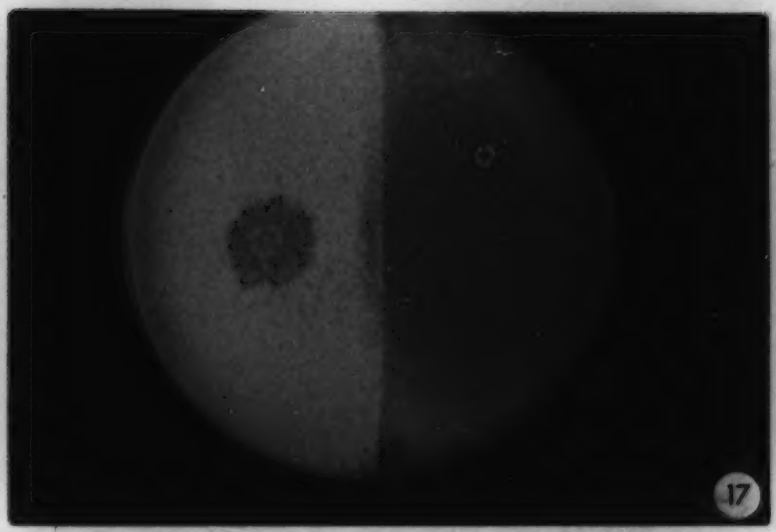
17

Table 7. Vegetative compatibility among isolates of *M. citricolor* grown on PDA. Cultures were incubated at 22°C under normal light conditions in the laboratory for one month. C=compatible, I=incompatible, IM=intermediate.

Isolates	2	7	10	3	9a	9b	9c	13	14	15	17	4	6a	6b	6c	6d	16	8a	8b	8c	8d	11	12	5a	5b	5c	ae5a	ae5b	ae10	ae11	ae13	ae14	aeF
2	C	C	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	I	IM	IM	IM	IM	I	IM	I	I	I	IM	IM	IM	IM	IM	IM	IM
7	C	C	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	I	IM	C	IM	IM	I	IM	I	I	I	IM	IM	IM	IM	IM	IM	IM
10	I	I	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	IM	I	IM	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM
3	IM	IM	IM	C	IM	IM	IM	IM	C	IM	IM	IM	I	I	I	I	IM	IM	IM	IM	IM	I	I	IM	IM	IM	IM	I	IM	IM	I	IM	IM
9a	IM	IM	IM	IM	C	C	C	IM	IM	C	IM	IM	IM	IM	IM	IM	I	I	IM	IM	I	IM	IM	I	IM	IM	I	IM	IM	I	IM	I	IM
9b	IM	IM	IM	IM	C	C	IM	C	C	C	IM	IM	IM	IM	I	I	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM
9c	IM	IM	IM	IM	C	IM	C	C	C	C	IM	IM	IM	IM	I	I	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM
13	IM	IM	IM	IM	IM	C	C	C	IM	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM	IM	I	IM	I	IM
14	IM	IM	IM	C	IM	C	C	IM	C	C	IM	IM	I	I	IM	IM	IM	IM	IM	IM	IM	I	IM	I	IM	I	I	IM	IM	I	IM	I	IM
15	IM	IM	IM	IM	C	C	C	C	C	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	I	IM
17	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	IM	PM	C	I	IM
4	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	C	I	I	I	I	I	IM	IM	IM	IM	I	I	I	IM	IM	IM	I	I	I	IM	IM	IM	IM
6a	I	I	I	I	IM	IM	IM	IM	I	IM	IM	I	C	IM	IM	IM	I	I	I	I	I	I	IM	I	I	I	I	I	IM	I	IM	IM	IM
6b	I	I	I	I	IM	IM	IM	IM	I	IM	IM	I	IM	C	IM	IM	I	I	I	I	I	IM	IM	I	I	I	I	I	IM	I	IM	IM	IM
6c	I	I	IM	I	IM	I	I	IM	IM	IM	IM	I	IM	IM	C	IM	I	I	I	I	IM	IM	IM	I	I	I	I	I	IM	I	IM	IM	IM
6d	I	I	I	I	IM	I	I	IM	IM	IM	IM	I	IM	IM	IM	C	I	I	I	I	IM	I	IM	I	I	I	I	I	IM	I	IM	IM	IM
16	I	I	IM	IM	I	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	IM
8a	IM	IM	I	IM	I	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	IM	C	IM	IM	IM	I	IM	I	IM	IM	IM	IM	IM	IM	I	IM	IM
8b	IM	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	IM	IM	C	C	IM	I	IM	I	IM	IM	IM	IM	IM	IM	I	I	I
8c	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	IM	IM	C	C	IM	I	IM	I	IM	IM	IM	IM	IM	IM	I	I	IM
8d	IM	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	I	I	I	IM	IM	IM	IM	IM	C	I	IM	I	IM	IM	IM	IM	IM	IM	I	IM	IM	IM
11	I	I	IM	I	IM	I	IM	I	I	IM	IM	I	I	I	IM	I	IM	I	I	I	I	C	IM	I	I	I	I	IM	IM	IM	I	IM	IM
12	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	C	I	IM	IM	IM	IM	IM	I	I	I	IM
5a	I	I	IM	IM	I	IM	IM	IM	I	IM	IM	IM	I	I	I	I	IM	I	I	I	I	I	I	C	IM	IM	IM	IM	IM	I	IM	IM	IM
5b	I	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	IM	IM	IM	IM	IM	I	IM	IM	C	C	IM	IM	IM	IM	I	IM	IM
5c	I	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	IM	IM	IM	IM	IM	I	IM	IM	C	C	IM	IM	IM	IM	I	IM	IM
5.3	I	I	IM	IM	IM	IM	IM	IM	I	IM	IM	IM	I	I	I	I	IM	IM	IM	IM	IM	I	IM	IM	C	C	IM	IM	IM	I	IM	IM	IM
ae5a	IM	IM	IM	IM	I	I	I	I	I	I	I	I	I	I	I	I	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	C	I	I	I	IM	I	IM
ae5b	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	C	IM	I	IM	I	IM
ae10	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	C	C	IM	IM	I
ae11	IM	IM	IM	IM	I	IM	IM	I	I	IM	IM	IM	I	I	I	I	IM	IM	IM	IM	IM	I	IM	I	I	I	I	I	C	C	I	I	I
ae13	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	C	IM	IM	IM	IM	IM	I	I	I	I	IM	I	I	IM	IM	IM	IM	IM	IM	I	C	I	I
ae14	IM	IM	IM	IM	I	I	I	I	I	I	I	I	IM	IM	IM	IM	IM	IM	I	IM	IM	I	I	IM	IM	IM	I	I	IM	I	I	C	I
aeF	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	IM	IM	IM	IM	IM	IM	IM	IM	IM	I	I	I	I	C

not possible to organize them in interaction groups. The only group of isolates that were highly incompatible when paired with the remaining isolates, were MCH6a, MCH6b, MCH6c and MCH6d.

Microscopic observation of hyphal interaction among non-sibling pairings were difficult to assess since two of the single basidiospore cultures had clamp connections. However, when the presumptive monokaryons, ssMCH5a and ssF, were paired the hyphae formed in the interaction zone showed clamp connections. It was also observed that the hyphae at the growing front seldom anastomosed, however, in the older part of the colony, anastomosis between and within the hyphae of the two isolates occurred, although at a very low frequency.

E. Lesion size and reaction of some coffee varieties to artificial inoculation

Lesions started to appear on the inoculated leaves as small brownish to black spots after three or four days of inoculation. After removing the plastic bags, they became lighter in colour with well defined margins (Figure 18). No gemmae or mushrooms ever formed on the lesions. This is normal when the plants are exposed to the sunlight.

All the isolates of *M. citricolor* were able to cause infection regardless of the amount of oxalic acid produced in culture.

Analysis of variance (Table 8) showed that the differences among isolates and varieties are significant, as well as the interaction between these two variables. Isolates behaved differently depending on the coffee variety used for inoculation. All the varieties were susceptible to varying degrees (Table 9).

Isolates from two red coloured lesions (MCH12 and MCH15) and two black lesions (MCH4 and MCH7) were included in the present study. The results of this test indicate that these isolates did not show any particular behavior with respect to lesion size and that they were very similar to the rest of the isolates.

Figure 18. Inoculated coffee leaf showing lesions caused by *M. citricolor*. Note the circular shape of the spots and the well defined margins.

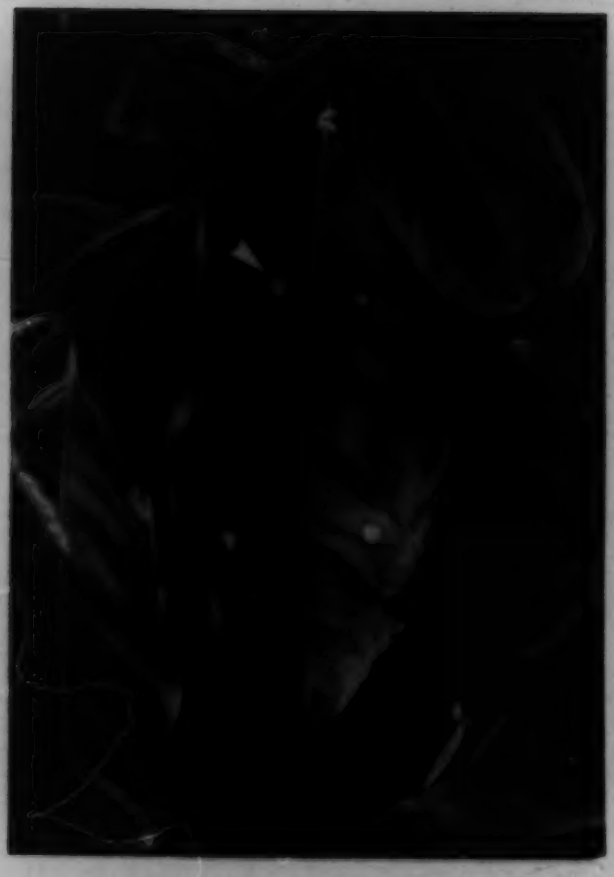

Table 8. Analysis of variance for lesion size caused by the 31 isolates of *M. citricolor* when artificially inoculated on eight coffee varieties.

Source	D.F.	SS	MS	F
Isolates (I)	30	30.76	1.03	5.85*
Varieties (V)	7	11.10	1.59	9.05*
I * V	210	36.82	0.18	5.29*
Residual	3472	114.97	0.03	

* Significantly different at P = 0.05

F. Cluster analysis

Cluster analysis showed that isolates can be separated into two well defined groups (Figure 19, see Table 1 for isolate identification). One group included all the single basidiospore cultures and the isolate from *C. liberica* while the rest of the field isolates were in another group. Within each of these two groups, isolates with similar characteristics clustered together before clustering with isolates from another group.

Cluster analysis confirmed the observation that the black and red lesions can not be separated into well defined groups, different from the rest of the isolates that came from tan coloured lesions. Further more, MCH7 (#11), which was isolated from a black lesion, had more characteristics in common with MCH12 (#21) which came from a red lesion (they clustered together), than with MCH4 (#3), which is the other isolate that was isolated from a black lesion.

Table 9. Lesion size (cm) formed by the 31 isolates of *M. citricolor* inoculated on eight coffee varieties.

	Varieties							
Isolates	8664	8667	8657	8660	Villalo.	Bourb.	Catur.	Cat. Rojo
H2	0.50*	0.53	0.64	0.48	0.33	0.40	0.64	0.48
H3	0.32	0.46	0.54	0.41	0.42	0.48	0.61	0.44
H4	0.43	0.38	0.71	0.44	0.28	0.51	0.48	0.61
H5a	0.36	0.50	0.47	0.46	0.37	0.40	0.45	0.61
H5b	0.43	0.37	0.59	0.38	0.51	0.46	0.34	0.62
H5c	0.54	0.47	0.55	0.48	0.30	0.60	0.63	0.46
H6a	0.54	0.35	0.40	0.50	0.52	0.42	0.68	0.89
H6b	0.42	0.35	0.38	0.46	0.29	0.30	0.42	0.60
H6c	0.35	0.30	0.40	0.44	0.36	0.34	0.49	0.51
H6d	0.41	0.46	0.43	0.48	0.43	0.37	0.51	0.69
H7	0.74	0.81	0.44	0.57	0.36	0.59	0.48	0.61
H8a	0.47	0.44	0.69	0.60	0.26	0.35	0.32	0.43
H8b	0.28	0.27	0.38	0.38	0.28	0.37	0.49	0.64
H8c	0.34	0.39	0.48	0.43	0.48	0.47	0.44	0.42
H8d	0.25	0.25	0.36	0.30	0.22	0.36	0.47	0.53
H9a	0.29	0.37	0.58	0.48	0.36	0.44	0.46	0.74
H9b	0.30	0.30	0.34	0.30	0.38	0.40	0.44	0.55
H9c	0.36	0.36	0.58	0.60	0.49	0.60	0.72	0.83
H10	0.30	0.90	0.32	0.70	0.51	0.42	0.50	0.52
H11	0.50	0.46	0.62	0.50	0.43	0.31	0.41	0.45
H12	0.31	0.42	0.44	0.33	0.51	0.33	0.43	0.57
H13	0.44	0.46	0.34	0.68	0.29	0.44	0.33	0.49
H14	0.41	0.45	0.70	0.48	0.34	0.24	0.29	0.53
H15	0.71	0.52	0.64	0.39	0.39	0.24	0.53	0.32
H16	0.43	0.45	0.55	0.48	0.40	0.56	0.48	0.96
H17	0.43	0.39	0.44	0.43	0.41	0.42	0.53	0.54
ssH5a	0.07	0.28	0.26	0.12	0.23	0.16	0.09	0.14
ssH9b	0.11	0.27	0.33	0.21	0.15	0.09	0.13	0.58
ssH11	0.39	0.26	0.42	0.26	0.26	0.12	0.20	0.37
ssH13	0.44	0.20	0.23	0.24	0.37	0.61	0.55	0.54
ssH14	0.49	0.34	0.35	0.46	0.05	0.07	0.16	0.28

* Each value represents a mean of 15 repeats.

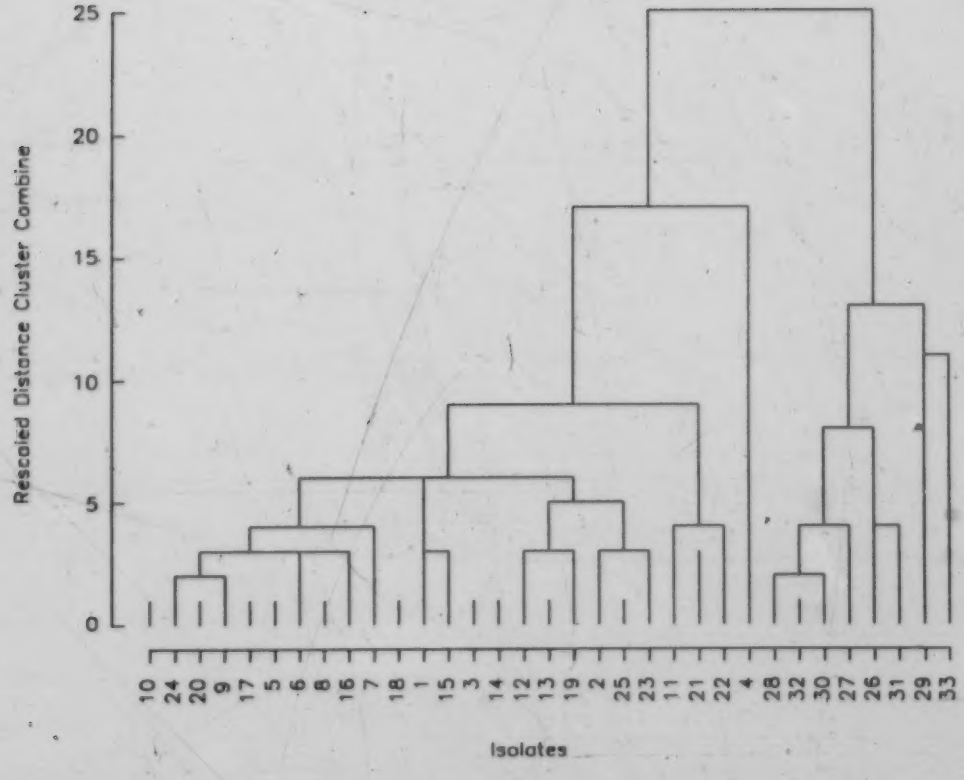

Figure 19. Dendogram using average linkage between the continuous variables gemmae, growth, oxalic acid, mycelium dry weight. Lesion size, vegetative compatibility and mushroom production were not included.

Chapter IV

Discussion and Conclusions

All species undergo changes continuously and slowly. These changes are always directed towards the conservation of the life form, for changes that will hamper it are eliminated (Kenaga, 1974). All pathogens, as living organisms, are able to produce variants and thus, are able to develop new capabilities of growth habits. However, most of the changes are undetected, simply because they are small, difficult to observe or even lethal.

When looking at cultures of *M. citricolor* isolated from diseased coffee leaves, it became evident that there are differences in colony morphology. Some grew very fast, forming large colonies with abundant gemmae, others grew very fast with no or very few gemmae and some others formed small colonies with or without gemmae (Figure 7). This was the first observation that suggested the presence of different strains in this fungus.

When colony size and gemma production were evaluated, differences among isolates were found to be significant (Tables 2 and 3). However, it was not possible to arrange isolates with similar characteristics into well defined groups since the differences among a series of isolates were small. In the case of *Fusarium oxysporum* Schlecht f. sp. *apii*, small colony size has been used to identify the virulent race 2, whereas avirulent strains formed large sized colonies (Correll *et al.*, 1986a; Puhalla, 1984). Besides, morphological variations may depend on external factors such as media and environment. Indeed, colony size was larger when isolates were grown on natural PDA and gemmae formation, although not affected by media, has been reported to be dependent upon light (Buller, 1934, Rodriguez, 1965). This indicated the need of additional information such as the ability to produce oxalic acid, pathogenicity, and vegetative compatibility, in order to attempt to group isolates with similar characteristics.

Oxalic acid is one of the most commom organic acids found in nature and its presence has been detected in soils, plants, microorganisms, etc. (Hodgkinson, 1977). The occurrence of this acid in fungi was reported as early as 1877 by Hamlet and Plowright, and its

involvement in pathogenesis was first considered by de Bary in 1886 when working with diseases caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (Ikotun, 1984). After this first observation, several reports dealing with the role of oxalic acid in pathogenesis have been published.

The presence of calcium oxalate crystals in cultures of *M. citricolor* was first observed by Ashby in 1925. In 1985, Rao *et al.* confirmed, using light and scanning electron microscopy, the presence of calcium oxalate crystals in the culture medium and along the growing hyphae. Further studies (Rao and Tewari, 1987) indicated that they were also present at the site of infection and in the necrotic tissue. Scanning electron microscope X-ray energy dispersive microanalysis, ^{13}C-nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectroscopy showed that the crystals were composed of calcium oxalate. These results indicated the capacity of *M. citricolor* to produce oxalic acid and suggested a key role for oxalic acid in the pathogenesis by this fungus.

The time course experiment to determine the best incubation period for oxalic acid production by *M. citricolor* showed that oxalic acid concentration followed the growth curve of the fungus up to day 12 and then decreased even though the fungus continued its growth (Figures 8 and 9). Similar results were obtained by D.V. Rao and J.P. Tewari (unpublished information) when working with the same fungus and by Maxwell and Lumsden (1970) with *S. sclerotiorum*. A possible explanation for this behavior is that *M. citricolor* may be metabolizing the oxalic acid after a certain incubation period.

It is known that oxalic acid can be oxidized to carbon dioxide and hydrogen peroxide or decarboxylated to yield formic acid and carbon dioxide (Hudgkinson, 1977; Franceschi and Horner, 1980). The enzymes required for these reactions have been found in fungi: oxalate oxidase in *Tilletia controversa* Kühn (Vaisey *et al.*, 1961), and oxalate decarboxylase in the wood destroying fungus *Collybia velutipes* (Curt. ex Fr.) Kummer (Shimazono, 1955) and *Myrothecium verrucaria* (Alb. & Schw.) Ditm (Lillehoj and Smith, 1965) and in certain strains of *Aspergillus niger* van Tiegh. (Emiliani and Bekes, 1964).

Variations in oxalic acid concentration were related to the pH values of the medium. As oxalic acid concentration increased, there was a drop in the pH, especially during the first 4 to 6 days. After day 12, when oxalic acid concentration started to decrease, a slight pH increase was observed (Figure 10). During the 14 days of this preliminary experiment, *M. citricolor* continued to grow although the growth was reduced towards the end of this period except for MCH14.

Based on preliminary experiments, the isolates were grown in PDB for 12 days and the amount of oxalic acid produced was determined. All the isolates, except for ssF revealed oxalic acid production ; besides, a great deal of variation in oxalic acid production was observed among isolates. Some of them produced twice as much as the others (Table 5).

As mentioned earlier, *M. citricolor* is a highly nonspecific plant pathogen. Its hosts include both monocotyledonous and dicotyledonous plants, as well as ferns (Sequeira, 1958). This ability to infect such a wide variety of plants may well be related to its capacity to produce oxalic acid, which is a non-host specific phytotoxin that affects the hosts cells by lowering the pH and by chelating the calcium from the middle lamella and rest of the cell wall. A key role for oxalic acid in pathogenesis has been established in several fungi such as *Sclerotium rolfsii* Sacc. (Higgins, 1927; Kritzman *et al.*, 1977; Punja and Jenkins, 1984), *S. sclerotiorum* (Maxwell and Lumsden, 1970; Lumsden and Dow, 1972; Noyes and Hancock, 1981), *Endothia parasitica* (Murr.) And. (Havir and Anagnostakis, 1983, 1985), *Leucostoma cincta* (Pers.: Fr.) Hohn and *L. personii* (Nits.) Hohn (Trauquair, 1987) and in *S. cepivorum* Berk. (Mankarios and Friend, 1980; Stone and Armentrout, 1985). Most of these fungi share with *M. citricolor* the capacity to cause diseases in a wide range of plant species.

Beside oxalic acid, many fungi are able to produce cell wall degrading enzymes that will further desintegrate the host tissue. Polygalacturonase and cellulase show a synergistic effect with oxalic acid; oxalic acid lowers the pH of the host tissue to levels optimum for enzyme activity and sequesters calcium from the host, thus conditioning the cell wall for hydrolysis (Bateman and Beer, 1965; Ikotun, 1984; McCarroll and Thor, 1985; Punja, 1987 and Punja *et al.*, 1985). Pectin degradation is considered to be the primary event leading to

loss of structure in the host tissue prior to invasion of the pathogen (McCarroll and Thor, 1985).

Pathogenicity tests showed that all the isolates of *M. citricolor*, irrespective of the amount of oxalic acid produced, were able to infect coffee leaves. However, the size of the lesions varied from one isolate to another (Table 8). Since it was impossible to inoculate all the isolates, or even one single isolate on all the coffee plants in one day, some of the variation observed among isolates might be due to the environmental variations, although this effect was attenuated as much as possible by covering the inoculated leaves with white plastic bags.

After removing the plastic bags, leaves became more exposed to environmental conditions. Both relative humidity and temperature during the months when the work was being carried were quite constant, with only slight variations (Appendix 1). Therefore, the effect of variations in environmental conditions on lesion size, if any, was minor or negligible.

There is a low but statistically significant correlation between lesion size and oxalic acid production. Such a correlation has been established for *S. sclerotiorum* (Marciano *et al.*, 1983, Maxwell and Lumsden, 1970) and *S. rolfsii* (Punja *et al.*, 1985). Actually, when a serial dilution of authentic oxalic acid solutions were placed on prescratched coffee leaves, chocolate-brown necrotic lesions, similar to those caused by *M. citricolor* were formed. The lesion size was proportional to the concentration of the acid applied (Rao and Tewari, 1987).

The single spore isolate ssF was not included in the field inoculations since it never formed gemmae. However, an interesting feature of this isolate is that it was the only isolate that did not produce oxalic acid. Based on previous unpublished results, where inoculation of coffee leaves using mycelial plugs has been successful, injured and uninjured leaves were inoculated with ssF mycelial discs under laboratory conditions. No lesions ever developed, which suggested that oxalic acid was fundamental for *M. citricolor*'s pathogenicity and in the absence of this acid, the pathogen was not able to infect the host. However, since inoculation was not done using gemmae and the isolate was not a hypovirulent strain derived from a virulent one, this evidence is not as firm as in the case of *E. parasitica*, where hypovirulent

strains obtained from virulent ones produced no oxalic acid. When inoculated on chestnut trees, they grew very little and for a short period of time (Havir and Anagnostakis, 1983). The capacity of these hypovirulent strains to cause infection to a small extent, might be due to the presence of cell wall degrading enzymes, mainly polygalacturonase. Even when there is a synergistic effect between oxalic acid and this enzyme, polygalacturonase itself can account for some of the symptoms observed (McCarroll and Thor, 1985).

In the case of *M. citricolor*, macerating-enzyme activities, specifically those of polygalacturonase and cellulase are low and reach peak levels 32 days after incubation of the fungus in a cell wall medium (D.V. Rao and J.P. Tewari, unpublished results). Such a delay in the onset of polygalacturonase activity indicates that calcium sequestration by oxalic acid plays a major role in tissue disintegration. In fact, the lesions caused by *M. citricolor* have well defined margins and no tissue disintegration is evident, indicating the absence of any appreciable macerating enzyme activity.

The sexual stage on *M. citricolor* is a small bright agaric that is seldom formed under field conditions, as opposed to the gemmae which are the actual means of dissemination of the fungus. In most cases, the basidiocarps have been obtained from infected plant material kept in moist chambers (Buller, 1934; Carvajal, 1939) or on diseased material undergoing decomposition in the field (Dennis,1950). Wellman (1950) spent over two years searching for them in many coffee plantations in Guatemala, Costa Rica and Colombia under different conditions, with no success.

Ashby (1925) and Buller (1934) were able to obtain basidiocarps on 2-3 weeks-old pure cultures grown on a bread and water medium (one part of bread:four parts of water) but in the present study, this was not always the case, since very few isolates produced them in monoculture. In cases where they were found, the cultures were 4-5 weeks-old and their formation was not consistent, even when all the conditions were kept constant. Salas and Hancock (1972) faced the same problem while studying the ability of some field isolates of *M. citricolor* to produce basidiocarps in monoculture and in co-culture with several fungi based on a chance observation that a *Penicillium* contaminant influenced their formation. When *M.*

citricolor was grown in co-culture with these fungi, basidiocarps were produced near the zone of contact between the two colonies which indicated that the basidiocap formation by *M. citricolor* was stimulated by the inducer fungi. Salas and Atkins (1970) tried to characterize the substance(s) produced by *P. oxalicum*. They found that the factor was dializable, heat stable, destroyed by acid hydrolysis but fairly stable to alkaline hydrolysis and with a molecular weight between 100 and 300, but the final identification of the factor was not made.

Based on Salas and Hancock's results, it was decided to determine the capacity of the isolates used to form the perfect stage when grown in co-culture with inducer fungi. The most active inducer fungus was *A. alternata*, although *T. flavus* and *Penicillium* sp. also showed some activity (Table 6). Forty-two percent of the isolates were not able to form basidiocarps, 31% formed sterile basidiocarps and only 27% formed fertile basidiocarps. The results indicate that basidiocarp production by *M. citricolor* can be induced by association with other microorganisms but the capacity of individual microorganisms to induce the sexual stage may vary. This situation may also be applicable under field conditions, besides, some environmental requirements have to be met, such as high humidity and presence of light. The effect of temperature on basidiocarp induction has, so far, not been studied.

When a basidiocarp that was actively discharging basidiospores, was placed over wounded and unwounded coffee leaves, no infection was observed. This confirms a previous report by Sequeira (1952) who used a light suspension of basidiospores to inoculate coffee leaves with no indication of infection. It might be that, as in the case of *S. sclerotiorum* ascospores, for infection to take place, the basidiospores may need, besides water, a nutrient source. If only water is available, the ascospores of *S. sclerotiorum* will germinate but no infection takes place (Abawi and Grogan, 1979). Although the basidiospores themselves were not capable of causing infection, gemmae produced by single spore cultures were infective.

It is not known if the basidiospores are able to infect other hosts and because the basidiocarps appear to be rare under natural conditions, the role of the perfect stage in *M. citricolor*'s life cycle remains unclear. On the other hand, gemmae are produced in large

numbers and are very effective as reproductive organs which makes the fungus less dependent on the sexual stage.

Two single basidiospore cultures (ssMCH13 and ssMCH14) used in this study showed clamped hyphae which is an indication of a dikaryotic condition. Sequeira (1954) performed some cytological studies on the nuclear cycles in the basidia and basidiospores of *M. citricolor* and found three different patterns of nuclear behavior. The young basidium had two nuclei that fused and underwent meiosis to form four nuclei. From this point on, three different patterns were observed to take place associated with the third (mitotic) division. In the typical pattern, which was the most common, four uninuleate basidiospores were produced and four residual nuclei remained in the basidium. In the atypical cycle A, there was a migration of two nuclei in each basidiospore. This pattern is characteristic of *M. muricata* Murr., where all the daughter nuclei migrate, two in each basidiospore (Smith, 1934). In an another alternative behavior, the atypical B, the third division did not occur until the four nuclei, produced in meiosis, had migrated into the basidiospores. This process has also been observed in *Schizophyllum commune* Fr. (Ehrlich and McDonough, 1949).

Upon germination of the basidiospores formed from the typical and atypical B nuclear patterns, the primary mycelium will not have clamp connections. It is believed that most of the isolates used in this study belong to these two groups. However, if two compatible nuclei migrate into one basidiospore as in the atypical A pattern, the resulting mycelium will show clamp connections. This situation perhaps prevailed in the two single basidiospore isolates with clamped hyphae (ssMCH13 and ssMCH14).

In *Athelia rolfsii* (Curzi) Tu & Kumbrough, clamp connections are reported to be present in all field isolates, as well as, in single basidiospore cultures (Punja and Grogan, 1983a). The mechanism behind this condition is not clear although it might have resulted from a pattern similar to what Sequeira (1954) called as atypical cycle A (Punja and Grogan, 1983a).

The absence of clamp connections on the mycelium of single basidiospore cultures has been used to distinguish them from parental isolates, but in *M. citricolor*, this criterion can

not be used for this purpose, since both field isolates and single basidiospore cultures can form clamp connections.

When the single basidiospore strains were paired in all possible combinations to check for heterokaryon formation, only in one case clamped hyphae were formed after their interaction. The rest of the pairings did not show any change, although those where ssMCH13 and ssMCH14 were used, it was difficult to assess because the hyphae of these isolates already have clamp connections.

Differences in virulence, morphological characteristics, or ability to form the perfect stage among isolates, can be influenced by environmental conditions. Because of this, these characteristics are often inadequate to be used alone to group isolates with similar traits. A very helpful test is the assessment of the extent to which hyphal anastomosis occurs, or the extent to which vegetative incompatibility or antagonism is manifested. This test is considered to be one of the easiest and most reliable methods of grouping genetically similar isolates (Correll *et al.*, 1986b; Puhalla, 1979, 1985; Stenlid, 1985).

When two genetically different mycelia are paired, the most evident manifestation of somatic incompatibility is the development of a reaction zone which has been termed as barrage, demarcation line, interaction zone, black line or inhibition zone. (Coates *et al.*, 1985; Rayner and Todd, 1979). Typically, this zone contains sparce mycelium, sometimes with hyphal disruption and often production of pigment within the hyphae and/or medium. The reaction is very variable both within and between species, especially regarding the width of the inhibition zone, colour and intensity of the pigment and development of aerial mycelium on either side of the interaction zone (Coates *et al.*, 1985; Golstein and Gilbertson, 1981; Rayner and Todd, 1979; Rayner *et al.*, 1984). When two genetically compatible isolates are paired, the mycelium intermingles, so a continuous mycelial mat will be formed and no interaction zone will develop (Goldstein and Gilbertson, 1981; Puhalla and Hummel, 1983; Stenlid, 1985).

Attempts to group the isolates of *M. citricolor* based on the capacity of the hyphae to fuse when two of them were paired was not possible. The frequency of anastomosis was very low, even between hyphae from the same isolate. Therefore, efforts were made to group the

isolates based on whether or not the colony interactions were compatible when paired. In this case, grouping became very difficult because of the presence of a third type of reaction, besides the compatible and incompatible ones described before. In this intermediate reaction, there was no inhibition zone but each isolate appeared to maintain its individual integrity.

If all the pairings must be classified as compatible or incompatible, then the intermediate reaction should be considered as incompatible, since there was no intermingling of the mycelia. However, because there is no information about the genetics of heterokaryon incompatibility in *M. citricolor* this assumption has to be regarded with caution.

When trying to establish interaction groups where, within each of them all the isolates grow together when paired and no antagonism zone develops, the lack of consistency in the response of *M. citricolor* isolates became evident. For example, when MCH2 was paired with MCH7, the reaction was compatible (Table 7). On the other hand, MCH7 was compatible with MCH8b, but when MCH2 was paired with MCH8b, an intermediate reaction developed as opposed to the expected compatible reaction. These results suggest that more than one gene and/or allele are involved in determining the compatibility in each isolate. Actually, it has been suggested that the control of somatic incompatibility in the Basidiomycetes must be either polygenic, multiallelic or both (Puhalla and Spieth, 1985; Raper and Flexer, 1971; Rayner *et al.*, 1984).

Pairing among single basidiospore isolates and between single basidiospore isolates and the parental or non-parental isolates were, in most cases incompatible or intermediate. In other fungi, such as *Fomes cajandari* Karst. (Adams and Roth, 1967), *Tanatephorus cucumeris* (Frank) Donk. (Flentje and Stretton, 1964), *Phellinus weirii* (Murr.) Gilb. (Hansen, 1979) and *A. rolfsii* (Punja and Grogan, 1983b) the formation of an inhibition zone has also been observed in this pairings. Once again, the factors behind these types of reactions are not clear, except that a multiple allele incompatibility system may exist.

The fact that isolates showed a great deal of variation regarding compatibility reactions, breeding of coffee plants for resistance towards *M. citricolor* becomes more difficult. Search for coffee varieties resistant to the fungus has not been successful. Up until

now, all the commercial varieties have been and are susceptible, even though lesion size varies from one variety to another (Table 9). The new varieties to be released, with resistance towards coffee rust (*H. vastatrix*) are also susceptible to *M. citricolor*, which means that the disease will continue to be a problem in coffee production.

Because each individual experiment has its contribution in characterizing the isolates of *M. citricolor*, a cluster analysis was performed to integrate all the results and try to group similar isolates together. The results showed that there are two well defined groups (Figure 19), one of them including all the single basidiospore isolates plus the isolate from *C. liberica* and the rest of the field isolates formed another group (see Table 1 for identification of the isolates). This means that differences between field isolates and the single spore isolates are very large. Within each group, there was variation in measured characteristics but clustering occurred at much lower levels.

When collecting the diseased coffee leaves for isolation of the pathogen, the general impression was that colour of the lesion (red, black or tan), was an indication of the presence of different strains of *M. citricolor*. After performing the cluster analysis, the two isolates from black coloured lesions and the two from red lesions did not cluster together but each of them appeared in different groups, which leads to the conclusion that colour of the lesion does not indicate different strains. This study indicated that for each of the characteristics studied there were significant differences among the isolates. However, when considering all the criteria as a whole, there were no consistent groups of isolates.

Further studies to confirm the role of oxalic acid in the pathogenesis by *M. citricolor* should be done. Induction of avirulent strains from virulent ones are necessary, so that differences in reaction when leaves are inoculated will be due to oxalic acid production and not to other factors. In this particular case, the low levels of macerating enzymes will ease the interpretation of the results. Artificial inoculation of coffee plants under field conditions showed that lesions developed in all 8 varieties, but size of the lesions varied from one variety to another (Table 9). Tu (1985) and Noyes and Hancock (1981) found that tolerance of white beans and sunflower to *S. sclerotiorum* is associated with tolerance to oxalic acid.

Breeding for resistance in coffee needs to be done and it can be oriented towards tolerance of the plants to oxalic acid.

A major area that needs to be examined is that of the factors governing heterokaryon compatibility. This thesis has provided some evidence that it might be multigenic and/or multiallelic. The so called intermediate reaction observed when two different isolates were paired requires more attention. So far, there is no information which may enable one to decide as to whether or not it should be considered an incompatible reaction.

Finally, the role of the perfect stage in the life cycle of the fungus remains unclear. The inability of the basidiospores to infect coffee leaves was confirmed in this study but it is not known if they can infect other hosts that are usually present in coffee plantations as weeds. A survey on the ability of the basidiospores to infect these hosts may help to further understand their role.

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Appendix

Meteorological data collected during the months of July, August, September and October, 1987 at Barva, Heredia.*

July

Date	Temperature (°C)			Precip. (mm)	R. H. (%)
	Min.	Max.	Ave.		
1	18.8	25.8	21.7		79
2	19.2	27.7	22.2	38.0	80
3	19.2	27.1	22.0	8.0	80
4	18.6	26.3	22.2	7.0	79
5	18.5	27.3	22.2	2.0	81
6	19.5	26.4	22.3		80
7	18.0	26..8	21.8	33.0	86
8	18.7	26.7	22.9	1.5	86
9	19.3	28.2	23.9	9.0	84
10	19.3	27.8	24.2	9.0	80
11	20.0	28.6	23.2	9.0	80
12	19.5	28.1	22.1	9.0	83
13	19.7	28.3	22.7	5.0	80
14	19.3	25.9	22.6	8.0	82
15	21.0	28.4	24.2		80
16	19.4	27.8	22.5	15.0	81
17	19.2	27.4	22.1	13.0	83
18	18.8	25.6	21.1	14.0	86
19	18.3	27.1	21.4	14.0	86
20	18.9	27.7	22.0	8.0	85
21	19.3	26.2	22.4		80
22	19.8	27.2	23.5	2.0	80
23	19.3	26.8	22.0	34.0	88
24	19.9	28.3	22.8	3.0	82
25	19.0	27.9	21.8	3.0	84
26	19.2	27.2	22.8	3.0	84
27	19.1	29.5	21.9		82
28	19.4	28.6	21..9	13.0	83
29	18.4	27.7	21.8	65.0	89
30	17.7	26.5	21.8	37.5	85
31	18.3	24.9	21.0	3.0	89

August

Date	Temperature (°C)			Precip. (mm)	R. H. (%)
	Min.	Max.	Ave.		
1	18.6	27.3	21.7	6.0	85
2	17.8	25.8	20.4	7.0	89
3	18.4	25.6	21.0	30.0	90
4	18.7	26.2	20.7	56.0	88
5	18.4	27.0	21.9	23.5	85
6	18.5	27.0	21.1	23.5	86
7	18.0	26.0	20.7	54.0	86
8	17.8	26.7	21.1	10.0	83
9	18.8	27.4	22.1		79
10	19.5	28.0	22.4	30.0	82
11	18.8	26.0	22.9		81
12	18.6	27.2	23.3	5.0	86
13	17.4	26.0	21.4		84
14	18.8	26.2	22.4	30.0	86
15	20.9	28.5	23.0	12.0	86
16	21.0	28.6	23.8		84
17	20.0	28.8	23.6	1.0	84
18	20.8	27.8	23.7		84
19	19.0	27.0	22.6	32.0	89
20	18.6	26.2	22.4		86
21	21.3	28.7	23.2		80
22	21.1	28.3	23.9	8.0	87
23	19.7	27.4	23.1		81
24	20.9	28.6	22.0		84
25	19.9	27.0	23.0		79
26	18.4	26.3	21.4	20.5	85
27	19.7	26.6	22.2	23.0	86
28	18.9	27.8	22.4		83
29	20.0	28.1	23.5		83
30	20.6	28.8	23.8		81
31	19.4	27.3	22.3	1.0	79

September

Date	Temperature (°C)			Precip. (mm)	R. H. (%)
	Min.	Max.	Ave.		
1	18.0	26.8	22.1	3.0	78
2	18.7	26.2	21.9		83
3	18.2	27.0	20.8	16.0	88
4	18.8	27.6	21.4	54.0	86
5	18.2	26.8	22.4	4.0	80
6	19.3	27.5	22.2	4.0	83
7	19.0	27.9	23.3	8.0	79
8	19.1	27.1	23.4		78
9	18.9	26.7	21.6	14.0	89
10	18.9	27.0	22.4		80
11	18.6	27.7	22.8	5.0	78
12	19.2	28.3	23.3	9.0	80
13	19.4	27.4	23.5		80
14	19.5	27.8	23.5		79
15	18.0	26.4	21.9	9.0	79
16	18.8	26.0	21.5		87
17	18.7	26.1	22.8	9.0	83
18	18.2	26.2	22.4	4.0	83
19	20.1	27.9	23.5		81
20	18.9	27.6	22.1		89
21	19.2	26.9	21.5	11.5	89
22	18.4	27.3	22.1		81
23	18.0	26.6	21.5	31.5	85
24	18.0	26.3	22.3	17.5	82
25	18.8	26.3	22.1	6.0	82
26	18.4	26.6	21.4	6.5	85
27	18.0	17.7	22.7	6.0	80
28	18.9	27.1	23.2		79
29	19.0	27.8	22.9	12.5	79
30	19.0	27.9	21.8	12.0	85

October

Date	Temperature (°C)			Precip. (mm)	R. H. (%)
	Min.	Max.	Ave.		
1	17.9	26.5	21.9	29.5	84
2	17.8	26.6	21.4	28.0	85
3	17.8	26.2	21.3	28.0	86
4	19.0	26.3	21.4	30.0	89
5	18.9	28.1	22.2	45.0	91
6	19.2	27.6	21.6	24.5	86
7	18.8	26.8	21.9	1.0	84
8	19.7	28.5	22.7	8.5	85
9	18.7	27.9	21.6		81
10	18.2	26.2	21.9	6.5	85
11	19.0	26.6	21.9	7.0	86
12	18.0	26.1	21.7		84
13	18.6	27.6	23.0	6.0	79
14	18.1	27.0	22.4		78
15	18.8	27.9	22.8		78
16	18.6	27.8	22.9		78
17	19.1	26.7	21.9	6.0	79
18	19.2	28.3	22.9	35.0	84
19	19.8	26.9	21.6		82
20	19.7	28.2	23.6		82
21	18.4	26.9	21.7	4.4	85
22	18.8	26.9	21.7		83
23	19.8	28.3	23.2		79
24	20.0	26.8	22.5	26.5	82
25	20.1	28.6	23.7		78
26	18.8	26.8	21.9		79
27	18.0	27.7	21.6	4.5	84
28	19.4	27.9	21.4	27.0	86
29	18.0	26.7	22.0		82
30	18.8	27.9	21.8	16.5	85
31	18.5	26.3	22.0		80